Food Research

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A UNIVERSAL GUSTOMETRIC SCALE IN D-UNITS*[a]

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University of Illinois College of Medicine, Chicago[b]
and
Illinois Department of Public Health[c]

(Manuscript received January 4, 1953)

STATEMENT OF THE PROBLEM

Taste scales used in different disciplines today are so diverse that intercomparison of results is practically impossible. Even within the field of food research and food technology, we find that the tastes of foods, of beverages, and of water are measured by numerous different scaling systems, while the responses of the subject to the tastable substances are often measured on still other types of scales which are not related.

This situation may be compared to the days prior to the *mercury* thermometer. The many diverse methods of measuring temperature devised during the seventeenth century likewise hindered intercomparisons: Measurement by expansion was different for air, water, alcohol, or gas; such various subdivisions of the temperature scale were used as 12°, 96°, 100°; temperature was related by some to the temperature of the body, by others to freezing water or to a freezing salt and ice mixture. A thermometer that was universally adaptable did not become available until such standard features were secured as a dependable fluid (mercury), and fixed points of reference.

So, too, today taste scales are as numerous and as diverse as were thermometers in the early 1600's, and to add to the complication we also have psychological as well as physical stimulus scales. The net product is that confirmation of results often is not forthcoming; new research is therefore impeded; and the full biological significance of subjective responses to taste remains confused and indefinite. Committees set up to resolve order out of the chaos often end where they start *(85)*. The chief reasons for this predicament, as we see it, are the lack of standards of reference for both stimuli and responses related to taste, and the need for an organization of measuring methods.

To clarify this situation we have attempted to develop a unified gustometric scaling device.

But before describing this new method, we should first examine the characteristics of taste scales already extant. This examination will reveal in greater detail why we need a unifying taste-measuring scale. Each scale we examine may have been adequate for a narrowly defined purpose. But if our purpose is to construct a scale which is universally adaptable, we must consider all of the possible uses for such a scale, and search for the missing common denominator.

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[a] Presented at the Twelfth Annual Meeting of the Institute of Food Technologists, Grand Rapids, Mich., June 11, 1952.

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Divergent Characteristics of Contemporary Taste Scales. The characteristics of contemporary taste scales are demonstrated in 12 selected examples presented in Table 1. These taste scales for sucrose have been developed during the past 44 years in different countries, and have beeen used in the various disciplines (as shown in line 2). The same 12 taste scales have served also in analyzing a number of different biological manifestations within the different disciplines (as listed in line 3).

In examining these taste scales, we observe the following divergencies:

1. Concentrations are reported in several different terms: molar concentrations; $\frac{\text{Moles}}{1}$; grams in 100 cc.; "concentration"; "strength"; percent solution; and parts per million. Another important method of expressing concentration, not appearing in this list, is the "normal solution," used chiefly in studies with electrolytes, toxicants, and salts of heavy metals *(24, 35, 36, 42, 53)*.
2. Scales start and end at highly different concentrations.
3. Scales are sometimes numbered serially in increasing concentrations (scales 7, 8, 9); in another case, in decreasing concentrations (scale 3).
4. All scales start and terminate at different levels of concentration.
5. Some scales cover a small sector of fine dilutions (scale 1), while others cover small sectors of intermediate dilutions (scale 4).
6. Scales may start at very low levels (scales 6, 7, 8), or at high levels (scale 5).
7. Some scales cover a low range of dilutions; others include only the highest concentrations. Separate, unconnected islands of taste-testing are formed.
8. Some scales have wide class intervals; other scales have small class intervals, usually without a statement of the basis upon which the scale interval has been selected.
9. Some scales follow numerical progression (scales 10, 11), others are logarithmic (scales 4, 9, 12), while still others vary the pace of progression in different regions along the same scale.
10. Scales are often replaced by the use of a *single* concentration of the taste solution (or the crystal substance itself), as in the diagnosis of ageusia (scale 5), in the numerous tests for taste blindness, and more recently as the standard stimulus for the construction of psychological taste scales *(5, 55)*.

These differences in methods of scaling taste are not characteristic of sucrose scales alone. Since the time that Valentin, over a hundred years ago, first demonstrated that the thresholds for primary tastes can be measured by various dilutions with distilled water, taste scales have been chiefly individualistic. Valentin *(89)*, in reporting on the taste of dilutions of sugar and syrup (sweet), rocksalt (saline), sulfuric acid (sour), aloe extract and quinine (bitter), used various combinations of the following dilutions to the point of taste extinction: 1/10, 1/18, 1/31, 1/53, 1/83, 1/213, 1/323, 1/426, 1/740, 1/12,500, 1/33,000, 1/900,000, 1/1,000,000. These dilutions were not ordered; each dilution was arrived at by guess and by test, each according to the intensity and recognition or perception of a taste when compared with distilled water.

During the following century and up to the present date, thousands of taste tests on a large number of taste substances and chemicals in solution have been reported. This literature includes scaling of taste, flavor, odor, of the common chemical senses, and of the common sensitivities affecting gustatory responses in many forms of animal life. The taste problems

studied have also included numerous biological side-effects in the relation between taste and the many survival features of food-getting. But in practically all of this literature the problems were not concerned with scales as instruments. Dilutions were selected merely as the most convenient, or as the most appropriate to the nature and range of the particular test under consideration.

Even when dilution rates have been of some concern and have been specified for the different primary tastes, they have varied from ¼ *(8, 90)* to ⅓ *(39)* to ½ *(31, 44, 45, 78, 88)*, or combinations of these and other dilution or concentration rates. As concentrations, the rates vary from 1¼ to 2, 3, or 4 times. In some instances 1 g. of sucrose in 100 cc. has been used as standard *(5, 6, 55)*. Added to this inter-science and inter-test difference in scale interval is the attending disharmony brought about by the accompanying use of different types of stock solutions: saturated, molar, molal, normal, grams percent by weight, or grams percent by volume. Often stock solutions differing in composition are labeled merely "concentration." Even the important distinction for taste between the molar and the molal solution is not always observed. All of these factors, and others that will be shown, enter into the final bizarre effect—a confusion of tongues.

This investigator believes, however, that for the problem set forth in this paper where gustometric scaling is the chief concern, the series of 12 scales for sucrose (extracted from the literature as examples and presented in Table 1) reflects the major scaling difficulties to be found throughout the literature on the subject. For an extension of this literature (from which the examples in Table 1 were drawn), the reader is referred to additional sources where the same diversity in types of scales may be observed:

For all 4 primary tastes: *(5, 9, 12, 13, 17, 29, 31, 33, 39, 42, 52, 55, 79, 83, 88, 89).*
For the sweet taste, in addition to the 12 in Table 1: *(25, 35, 61).*
For the saline taste: *(15, 19, 20, 34, 49, 50, 59, 69, 71, 77).*
For the acid taste: *(7, 51, 60, 67).*
For the bitter taste: *(10, 20, 62, 65, 66, 91).*
For the multiple taste of PTC: *(4, 8, 14, 44, 45, 74, 78, 90).*
For the taste of various other chemicals classed as electrolytes, toxic substances, or salts: *(9, 24, 35, 36, 42, 48, 53, 83).*

Several of these sources refer in turn to scales used by other experimenters in previous reports.

In the development of the design of gustometric scales, these past-present difficulties, brought to light especially by comparative taste analysis, may serve as guide buoys for incorporating present needs and anticipating future applications.

DEVELOPMENT OF A UNIVERSAL GUSTOMETRIC SCALE IN D-UNITS

Let us now consider what would be required of a universally adaptable taste scale.

As already stated, a universal taste scale should encompass the various uses reported in the scientific literature, the latest developments in food technology, the diffuse array of subjective responses, and the uses projected for the future.

The composite result of this research analysis of taste scaling is a universally adaptable gustometric scale or taste scale, presented in streamlined form in Figure 1. In this one chart may be observed at a glance many of the comparative values and essential features of a universal gustometric scale.

TABLE 1

Examples of different chemical scales (for sucrose) used for the measurement of taste responses by investigators in different fields

	1		2	3			4	5	6
Date	1908		1919	1925			1928	1928, 1940	1940
Author	Lemberger *(54)*		Renquist *(71)*	Biester, Wood, and Wahlin *(7)*			Ufland *(88)*	Börnstein *(12, 13)*	Richter and Campbell (73) .
Department	Physiology		Physiology	Home Economics			Physiology, Occupational Diseases	Neurophysiology, Psychology	Psychobiology
Subject of investigation	Psychophysics of taste		Chemistry of taste	Physiological chemistry of carbohydrates			Taste thresholds of lead workers	Cortical representation of taste	Comparative taste thresholds
Description of solution	Concentration in %		Molar concentration as %	Grams per 100 cc.			"Strength of solution in %"	Grams in 100 cc.	Concentration in %
Measure	P, R	JND	R	Soln. No.	R	JND	R	R	P, R
	0.90	45.00	0.020	(1)	1.50	9.75	4.0	40.0	1.60
	0.80	35.00	0.030	(2)	1.25	8.25	2.0	10.0	1.50
	0.75	22.95	0.050	(3)	1.00	6.75	1.0	4.0	1.40
	0.70	18.75	0.075	(4)	0.75	5.25	0.5		1.30
	0.65	16.17	0.10	(5)	0.50	3.75			1.20
	0.60	13.90	0.20	(6)	0.25	2.25			1.10
	0.55	11.97	0.40			0.75			1.00
	0.50	10.35	0.50						0.90
	0.47	8.916	0.75						0.80
	0.45	7.75	1.00						0.70
	0.43	6.75							0.60
	0.40	5.83							0.50
		5.08							0.40
		4.39							0.30
		3.775							0.20
		3.267							0.10
		2.825							0.08
		2.45							0.06
		2.13							0.04
		1.85							0.02
		1.65							0.01
		1.40							
		1.125							
		0.82							
		0.60							
		0.44							

	7		8			9			10	11	12
Date	1941		1943			1947			1949	1950	1951
Author	Wilkes *(93)*		Bureau Human Nutrition and Home Economics *(17)*			Falconer *(31)*			Janowitz and Grossman *(50)*	Goetzl, Ahokas, and Payne *(38)*	Gould *et al. (39)*
Department	Psychology		Nutrition, Home Economics			Genetics			Clinical Medicine	Medical Research	Food Technology
Subject of investigation	Qualitative stimulus thresholds vs. disease		Taste judges			Taste inheritance			Physiology of appetite and hunger	Daily variation in taste	Taste effect of insecticides
Description of solution	Vol. mol. concentration in %		$\frac{\text{Moles}}{1}$			Parts per million			% Solution	% Solution	Molar concentration as %
Measure	Soln. No.	R	Soln. No.	R		Soln. No.	R		R	R	R
	(21)	0.032	(15)	0.15		(5)	2.	:1,000,000	1.0	2.5	0.146
	(20)	0.030	(14)	0.10		(4)	1.	:1,000,000	0.9	2.4	0.049
	(19)	0.028	(13)	0.075	H	(3)	0.5	:1,000,000	0.8	2.3	0.016
	(18)	0.026	(12)	0.05	i	(2)	0.25	:1,000,000	0.7	2.2	0.0054
	(17)	0.024	(11)	0.04	g	(1)	0.125	:1,000,000	0.6	2.1	0.0018
	(16)	0.022	(10)	0.035	h				0.5	2.0	0.0006
	(15)	0.020	(9)	0.03					0.4	1.9	0.0002
	(14)	0.018	(8)	0.025					0.3	1.8	
	(13)	0.016	(7)	0.02	M				0.2	1.7	
	(12)	0.014	(6)	0.015	e				0.1	1.6	
	(11)	0.012	(5)	0.01	d					1.5	
	(10)	0.01	(4)	0.0075						1.4	
	(9)	0.008	(3)	0.005	L					1.3	
	(8)	0.006	(2)	0.001	o					1.2	
	(7)	0.004	(1)	0.0005	w					1.1	
	(6)	0.002								1.0	
	(5)	0.001								0.9	
	(4)	0.0005								0.8	
	(3)	0.00025								0.7	
	(2)	0.000125								0.6	
	(1)	0.00005								0.5	
										0.4	
										0.3	
										0.2	
										0.1	

Fixed point of origin. Heretofore a common fixed point of origin, even for saporous substances as a group, has never been considered. And yet it will be seen that such a point is of first importance in the establishment of a universal scale encompassing both foods and subjects. The fixed point of origin must be based upon the characteristics of *centrality*, and of *permanence*, and should also be amenable to *ease in operations*—in diluting and concentrating, and in statistical and comparative analysis. Foods and chemicals in solution should have *identical* scales from the same fixed point.

Centrality. For operational advantages, the fixed point of origin should approximate centrality in relation to the universe of tastable substances and of the taste-sensing abilities of most organisms. The feature of centrality to foods and chemicals tasted by man, posited at *one concentration*, can be noted visually in Figure 1. Later in the discussion examples will be taken from the foods and responses of the human being, and reference made to animals and lower organisms.

A UNIVERSALLY ADAPTABLE GUSTOMETRIC OR TASTE METER

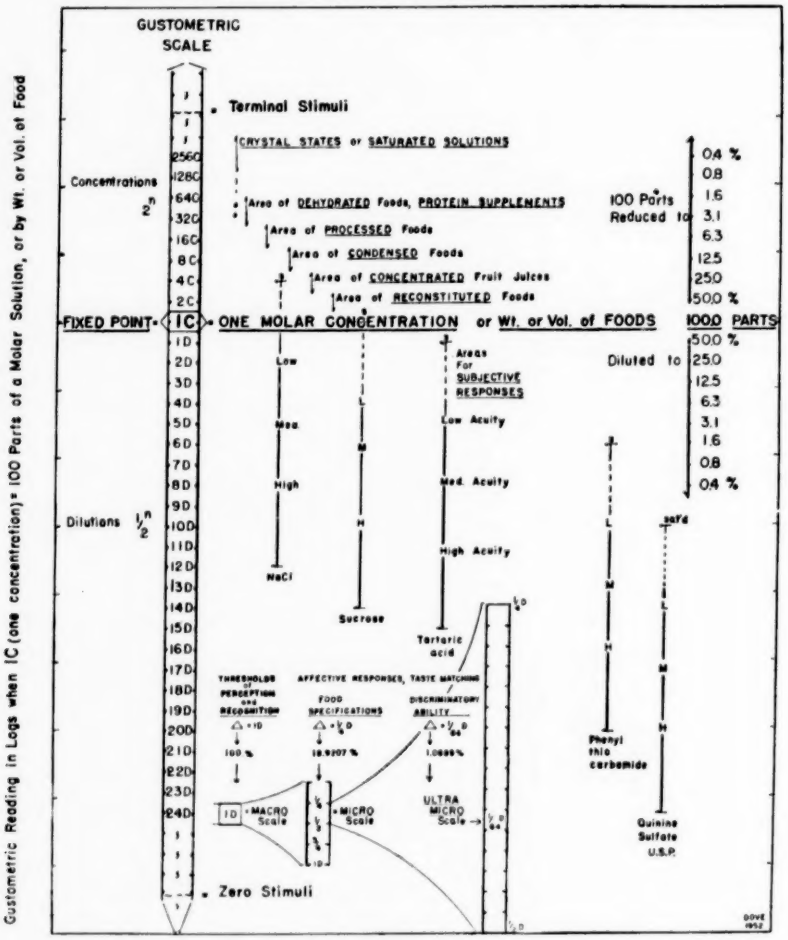

Figure 1

Permanence. As to permanence, the saturation point of a chemical in solution is unsuitable, because it varies with the different solvents used (whether water, alcohol, ether, etc.). The zero stimulus and the terminal stimulus are even less dependable; each of the 2 end-points of an intensity scale varies as a threshold from individual to individual, and the final stimulus level fixed upon by the experimenter must be an assumed value when applied to population groups. Nor can the crystal state be used as a fixed point because the taste response may be slower or at a lower taste intensity value for the crystal than for the same chemical already dissolved in a solvent.

According to the numerous requirements imposed upon the measurement of taste in all of its manifestations, regardless of the discipline concerned, the most permanent and central point of origin for chemicals in solution appears to be the *one molar solution.*

The law of constant proportions in chemical compounds and the establishment of International Atomic Weights based upon Oxygen [with a defined value of 16 as the standard reference weight *(92)*] provides, for practical purposes, a permanent taste reference value in the gram-molecular weight made up to 1000 cc. volume.

Ease in operation. When the solution is *made up to* 1000 cc. volume (the chemical compound is not added to 1000 g. of water, as in the molal solution), it lends itself with ease to calculations of concentrations at any dilution level.

Either the solution by "percent" as grams added to 100 cc. of solvent, or the molal solution as a gram-molecule (or mole) added to 1000 g. of solvent, does not lend itself to comparative taste advantages expected from dilution procedures; the reason is that different chemicals—and especially complex mixtures and foods—displace different quantities of the same solvent. The chemical in the molar solution is a *part of the whole;* while the chemical in the solution by an added "percent," or the chemical in the molal solution, is a *part added,* and the *new whole* becomes indeterminate. In some chemical processes this distinction is trivial and unimportant. In chemical taste scales, in food taste scales, and especially in the *reconstitution* of processed foods, these volume/weight relationships assume a significance of sufficient magnitude that they should be incorporated in the structure of the scaling device.

The central position of one concentration of the molar solution concurrently represents the origin to the series of *dilutions downward* and the origin to the series of *concentrations upward,* both on the same physical-chemical scale. In fact, the concentration series is merely an extension of the dilution series. Dilutions can be initiated at any point on the concentration series. *The entire series, including concentrations and dilutions, is therefore measurable in uniform dilution units regardless of stimuli or responses;* for that reason the scale is termed *a D-Unit scale.*

The identity between chemical D-Unit scales and food D-Unit scales. With the establishment of a fixed point of origin for chemicals in solution at one molar concentration by volume, its centrality and other important features are further demonstrated by the fact that the same scale can be used to measure natural foods. This *identity of scales* has many comparative advantages, as will be observed in the applications to be presented.

For any food item for which molecular weight is beyond calculation, we can use the natural food scale, in which the central fixed point of origin is the normal weight or volume, expressed in parts to the whole. Concentration on the scale *above* this point of origin opens up to an orderly gustometric analysis the whole field of dehydration and concentration of foods:

2 C = 50.00% (100 parts are dehydrated or reduced to 50 parts)
4 C = 25.00%
8 C = 12.50%
16 C = 6.25%
32 C = 3.13%
64 C = 1.56%, etc., as shown in the right-hand scale of Figure 1. Dilutions *below* the origin of 100 parts also serve as measurable degrees in the formulation of mixtures and recipes (a dilution procedure). Between the highly dehydrated or concentrated foods (64 C = 1.56%) and the dilution series, the entire range of *reconstitution* of dehydrated foods can be marked off for gustometric responses on the same scaling device.

By this scheme the important operations in food technology—dehydration, processing, condensing, reducing, concentrating, reconstituting, diluting, and formulation by

parts—are placed on the one and the same taste-measuring scale. The different products of these technological procedures take up different areas of the gustometric scale of Figure 1. Before the concentrated foods are ready for consumption, they are usually reconstituted (diluted) to levels slightly above, equal to, or slightly below the food in its natural state, which is the central fixed point of origin.

The scale interval. Of equal importance to the establishment of a central fixed point of origin, the scale interval must be carefully adjusted to meet many different situations. A quick glance over the wide range of demands placed upon different-sized scale intervals and different methods of progression makes one wish for a rubber scale. And that is what, figuratively, must be secured. Like the aperture and focus of a camera lens, the taste scale interval selected must be adjustable to universal focus. Furthermore it must be adjustable for use, let us say, as a reading glass, as a microscope, as a telescope, all three in one; and yet that is asking of it more than is expected of the telescope, which is not universal as a lens and cannot be used as a reading glass.

The abundance and diversity of taste-scaling methods now in use appear to indicate the widespread need for a taste focusing device that possesses universal features. In order to develop a scale with a universal taste focus, exploratory research has been made to find the different kinds of taste focus one might encounter in the universe of taste stimuli and responses.

Out of this research has come the conclusion that a universally adaptable scale interval can be secured from *the formula of* $\frac{1}{2}^n$ *and* 2^n, *including their successive fractions, when anchored to the central fixed point of origin of one molar solution by volume, and to natural weight or volume of tastable substances as* $\frac{1}{2}^n$ *and* 2^n *parts of the whole.*

This scheme for scaling gustatory responses has several important advantages:

First: The formula of $\frac{1}{2}^n$ and 2^n (achieved by halving and doubling the concentration) is amenable to both logarithmic and arithmetical progression from the central fixed point of origin, and from each dilution level. Since the discrimination of taste differences is not always proportional to the magnitude of the intensity of the stimulus *(1, 11, 40, 41, 58, 63, 87)*, the open mathematical feature of the scale provides operational advantages in determining the actual relationship existing between taste stimulus and response.

Second: The logarithmic scale of $\frac{1}{2}^n$ and 2^n can be fractioned readily at any point on the scale into successively smaller degrees of fineness to provide the universal taste focus required of a true gustometric scale. The fractions also may be arithmetical, logarithmic, or based upon any mathematical formula examined for adequacy in expressing the taste stimulus-response relationship. When the fractions are required as log units, increments may be secured by the following factors:

1	D =	100.0000000%
$\frac{1}{2}$	D =	41.4213563%
$\frac{1}{4}$	D =	18.9207115%
$\frac{1}{8}$	D =	9.0507733%
$\frac{1}{16}$	D =	4.4273783%
$\frac{1}{32}$	D =	2.1897148%
$\frac{1}{64}$	D =	1.0889286%

These fractions are universally applicable to any D- or C-Unit of any molar solution by volume and to any concentrated, diluted, or natural food or saporous substance treated mathematically and physically as part to the whole.

Third: Experience with the use of this scale by the writer reveals to date that gustometric data fall into taste-measuring focus in 3 specific degrees of fractionation. These 3 degrees of fractionation can be carried out by the use of the same fractions anywhere on the progression scale, as indicated in the bottom section of Figure 1.

Fractionation of the D-Unit scale. The 3 degrees of taste focus provided by fractionation of the D-Unit scale are measured by the Macro, the Micro, and the Ultramicro taste scales. Each of these 3 scales has important special uses, and will therefore be discussed in some detail.

The Macro taste scales. The Macro taste scales are equivalent to the D-Units of 100% magnitude, secured by halving or doubling the concentration. The D-Units are therefore 2 to 5 times larger than the reported taste responses measured in JND's, over

any part of the sucrose taste stimulus continuum *(7, 54)*. In addition to their value in the structure of the scaling device, these D-Units are also suited for use in preliminary scaling of taste thresholds, since the usual day-to-day fluctuation in taste thresholds *(9, 33, 78, 79)* may not at present warrant a finer scale interval for routine tests.

This D-Unit scale of ½° provides at least 5 or more class intervals for all 5 of the primary taste substances shown in Figure 1. Furthermore (according to dilution scaling represented in Figure 1) all 5 primary taste substances, i.e. sodium chloride, sucrose, tartaric acid, phenyl thiocarbamide, and quinine sulfate, can be placed conveniently on the same master D-Unit scale.

The values marked for High, Medium, and Low taste acuity have been based upon the first set of tests on approximately forty normal subjects. These arrays will form the basis of expected distributions by population groups. The lowest levels marked for each solution are approximately 2 units below the response of the most acute normal taster observed to date. The highest level marked for each solution usually represents the first D-Unit below easy saturation of the solute in water. The high mark on each scale can be raised by the use of a solvent more active than water, or by the use of higher temperatures *(80, 81)*. On the other hand, scales for any one of these solutes can be extended to lower dilutions, if and when needed and as far as desired.

The scale area between High acuity and Saturation is available for scaling hyposensitive tasters, levels of ageusia, or degrees of taste-blindness, and for recording affective and subjective responses. The dilutions below the lowest levels marked for each solution can be extended indefinitely for hypersensitve tasters, for any lower form of animal or insect life which might possess marked gustatory sensitivity or chemoreceptive response, and for detecting rancidity, reversion products *(2)*, and toxic substances *(39)* possessing taste qualities. Some of these substances can be detected at more dilute levels by taste than by the current chemical procedures *(3)*.

The scientific literature reporting threshold values for the 4 primary tastes is widespread and voluminous. The reported threshold values generally fall within the range secured by the writer (as presented in D-Units in Figure 1), but the average thresholds vary widely.

For example consider the various scales for sucrose thresholds. When recalculated into D-Units, they are found to span different ranges, for example: 4 D to 8 D *(7)*; 4 D to 15 D *(93)*; 2 D to 11 D *(17)*; 4 D to 9 D *(31)*; 5 D to 9 D *(50)*; 3 D to 9 D *(38)*; 2 D to 13 D *(39)*. On these different sucrose scales, the average threshold for man tends to vary, sometimes as much as 100% *(30, 73)*.

Similar examples of diversity in span of threshold scales might be presented for the other primary tastes. For instance in reports from 10 different investigators, the average threshold values for salt vary between 4 D and 7 D *(75)*. An interpretation of the genetic and metabolic relationship between genes responsible for the expression of individually different taste reactions to sucrose, salt, and phenylthiocarbamide may depend upon the type of dilution scale used, both as to range and as to degree of fineness of the class intervals used.

For comparative gustometries the D-Unit scale also provides a master scale for the taste records of animals, lower vertebrates, and insects. Threshold tests on the chimpanzee *(65)*, monkey *(10, 65)*, rat *(65, 72, 91)*, rabbit *(36)*, cockroach *(35)*, moth *(36)*, housefly *(27)*, blowfly, butterfly, water beetle, frog, and fish *(61)* demonstrate that when taste ability or chemoreceptability exists, the average threshold levels in these lower organisms, though generally less acute than in man, are frequently within the same D-Unit range as in man. This is true in spite of the great lack of uniformity among the results of different workers on the same species *(24, 36)*.

For the rabbit a sucrose threshold *(36)* recalculated into D-Units is close to 5 D with a standard error (combined + and −) of ¼ D. The sucrose acceptance threshold for 40 cockroaches in 160 tests *(35)*, restated in D-Units, provides a mean value between 5 and 6 D, with a maximum individual range from 3 D to 8 D. The values and ranges, it will be noted, are comparable to those secured for man, and also comparable to the range in response, whether gustative or chemoreceptive, for the blowfly, butterfly, and honey bee (see *35*).

In lower forms of animal life and in insects, response to some of the primary taste substances may be absent *(47)*. The response depends upon contact, the nature of the chemical, and the structure of the receptor surfaces *(28)*. Chemoreceptors may be

TABLE 2

Universal gustometric scales in ¼ log D-Units for 5 primary taste solutions and for natural and processed foods and beverages
(Scales span the range of dilutions from area of saturation to area of subliminal thresholds of perception of a difference in taste.)

Sodium Chloride NaCl M.W. = 58.454 IAW[d]		Sucrose $C_{12}H_{22}O_{11}$ M.W. = 342.296 IAW		Tartaric Acid $C_4H_6O_6$ M.W. = 150.088 IAW		Phenylthiocarbamide $C_6H_5 \cdot NH \cdot CS \cdot NH_2$ M.W. = 152.216 IAW		Quinine Sulfate, U.S.P. $(C_{20}H_{24}N_2O_2)_2 \cdot H_2SO_4 \cdot 2H_2O$ M.W. = 782.93 IAW		Foods and Beverages Natural or Processed Parts to the Whole		
Dilution Units (log)	Grams in liter of solution	Dilution Units (log)	Grams in liter of solution	Dilution Units (log)	Grams in liter of solution	Dilution Units (log)	Grams in liter of solution	Dilution Units (log)	Grams in liter of solution	Scale for Dilutions $1/2^n$	Volume or Weight	Scale for Concentrations 2^n
¾	393.2301	¾	575.6710	¼	126.2085	¼	4.0000	¼	1.2859			
½	330.6658	½	484.0796	½	106.1282	½	3.3635	½	1.0813			
¼	278.0557	¼	407.0608	¾	89.2429	¾	2.8284	¾	0.9092			
4 C	= *233.8160*	*1 C*	= *342.2960*	*1 D*	= *75.0449*	*6 D*	= *2.3784*	*10 D*	= *0.7646*	*1 C*	= *1,000.0000*	= *1 C*
¾	196.6150	¼	287.8355	¼	63.1042	¼	2.0000	¼	0.6429	¼	840.8964	
½	165.3329	½	242.0398	½	53.0641	½	1.6818	½	0.5406	½	707.1068	
¼	139.0278	¾	203.5304	¾	44.6214	¾	1.4142	¾	0.4546	¾	594.6036	
3 C	= *116.9080*	*1 D*	= *171.1480*	*2 D*	= *37.5229*	*7 D*	= *1.1892*	*11 D*	= *0.3823*	*1 D*	= *500.0000*	= *2 C*
¾	98.3075	¼	143.9177	¼	31.5521	¼	1.0000	¼	0.3215	¼	420.4482	
½	82.6664	½	121.0199	½	26.5320	½	0.8409	½	0.2703	½	353.5534	
¼	69.5139	¾	101.7652	¾	22.3107	¾	0.7071	¾	0.2273	¾	297.3018	
1 C	= *58.4540*	*2 D*	= *85.5740*	*3 D*	= *18.7610*	*8 D*	= *0.5946*	*12 D*	= *0.1911*	*2 D*	= *250.0000*	= *4 C*
¼	49.1538	¼	71.9589	¼	15.7761	¼	0.5000	¼	0.1607	¼	210.2241	
½	41.3332	½	60.5100	½	13.2660	½	0.4204	½	0.1352	½	176.7767	
¾	34.7570	¾	50.8826	¾	11.1554	¾	0.3535	¾	0.1137	¾	148.6509	
1 D	= *29.2270*	*3 D*	= *42.7870*	*4 D*	= *9.3805*	*9 D*	= *0.2973*	*13 D*	= *0.0956*	*3 D*	= *125.0000*	= *8 C*
¼	24.5769	¼	35.9794	¼	7 8880	¼	0.2500	¼	0.0804	¼	105.1121	
½	20.6661	½	30.2550	½	6.6330	½	0.2102	½	0.0676	½	88.3883	
¾	17.3785	¾	25.4413	¾	5.5777	¾	0.1768	¾	0.0568	¾	74.3254	
2 D	= *14.6135*	*4 D*	= *21.3935*	*5 D*	= *4.6903*	*10 D*	= *0.1486*	*14 D*	= *0.0478*	*4 D*	= *62.5000*	= *16 C*
¼	12.2884	¼	17.9898	¼	3.9440	¼	0.1250	¼	0.0402	¼	52.5560	
½	10.3333	½	15.1275	½	3.3165	½	0.1051	½	0.0338	½	44.1942	
¾	8.6892	¾	12.7207	¾	2.7888	¾	0.0884	¾	0.0284	¾	37.1627	
3 D	= *7.3067*	*5 D*	= *10.6968*	*6 D*	= *2.3451*	*11 D*	= *0.0743*	*15 D*	= *0.0239*	*5 D*	= *31.2500*	= *32 C*
¼	6.1442	¼	8.9949	¼	1.9720	¼	0.0625	¼	0.0201	¼	26.2780	
½	5.1667	½	7.5637	½	1.6583	½	0.0526	½	0.0169	½	22.0971	
¾	4.3446	¾	6.3603	¾	1.3944	¾	0.0442	¾	0.0142	¾	18.5814	

4 D	=	*3.6534*	*6 D*	=	*5.3484*	*7 D*	=	*1.1726*	*12 D*	=	*0.0372*	*16 D*	=	*0.0119*	*6 D*	=	*15.6250*	= *64 C*
¼		3.0721	¼		4.4974	¼		0.9860	¼		0.0312	¼		0.0100	¼		13.1390	
½		2.5833	½		3.7819	½		0.8291	½		0.0263	½		0.00845	½		11.0185	
¾		2.1723	¾		3.1802	¾		0.6972	¾		0.0221	¾		0.00710	¾		9.2907	
5 D	=	*1.8267*	*7 D*	=	*2.6742*	*8 D*	=	*0.5863*	*13 D*	=	*0.0186*	*17 D*	=	*0.00597*	*7 D*	=	*7.8125*	= *128 C*
¼		1.5361	¼		2.2487	¼		0.4930	¼		0.0156	¼		0.00502	¼		6.5695	
½		1.2917	½		1.8909	½		0.4146	½		0.0131	½		0.00422	½		5.5243	
¾		1.0862	¾		1.5901	¾		0.3486	¾		0.0110	¾		0.00355	¾		4.6453	
6 D	=	*0.9133*	*8 D*	=	*1.3371*	*9 D*	=	*0.2931*	*14 D*	=	*0.00929*	*18 D*	=	*0.00299*	*8 D*	=	*3.9063*	= *256 C*
¼		0.7680	¼		1.1244	¼		0.2465	¼		0.00781	¼		0.00251	¼		3.2848	
½		0.6458	½		0.9455	½		0.2073	½		0.00657	½		0.00211	½		2.7621	
¾		0.5431	¾		0.7950	¾		0.1743	¾		0.00552	¾		0.00178	¾		2.3227	
7 D	=	*0.4567*	*9 D*	=	*0.6686*	*10 D*	=	*0.1466*	*15 D*	=	*0.00465*	*19 D*	=	*0.00149*	*9 D*	=	*1.9531*	= *512 C*
¼		0.3840	¼		0.5622	¼		0.1233	¼		0.00391	¼		0.00126	¼		1.6424	
½		0.3229	½		0.4727	½		0.1036	½		0.00328	½		0.00106	½		1.3811	
¾		0.2715	¾		0.3975	¾		0.0872	¾		0.00276	¾		0.000888	¾		1.1613	
8 D	=	*0.2283*	*10 D*	=	*0.3343*	*11 D*	=	*0.0733*	*16 D*	=	*0.00232*	*20 D*	=	*0.000747*	*10 D*	=	*0.9766*	= *1024 C*
¼		0.1920	¼		0.2811	¼		0.0616	¼		0.00195	¼		0.000628	¼		0.8212	
½		0.1615	½		0.2364	½		0.0518	½		0.00164	½		0.000528	½		0.6905	
¾		0.1358	¾		0.1988	¾		0.0436	¾		0.001381	¾		0.000444	¾		0.5807	
9 D	=	*0.1142*	*11 D*	=	*0.1671*	*12 D*	=	*0.0366*	*17 D*	=	*0.001161*	*21 D*	=	*0.000373*	*11 D*	=	*0.4883*	= *2048 C*
¼		0.0960	¼		0.1405	¼		0.0308	¼		0.000977	¼		0.000314	¼		0.4106	
½		0.0807	½		0.1182	½		0.0259	½		0.000821	½		0.000264	½		0.3453	
¾		0.0679	¾		0.0994	¾		0.0218	¾		0.000691	¾		0.000222	¾		0.2903	
10 D	=	*0.0571*	*12 D*	=	*0.0837*	*13 D*	=	*0.0183*	*18 D*	=	*0.000581*	*22 D*	=	*0.000187*	*12 D*	=	*0.2441*	= *4096 C*
¼		0.0480	¼		0.0703	¼		0.0154	¼		0.000483	¼		0.000157	¼		0.2053	
½		0.0404	½		0.0591	½		0.0130	½		0.000411	½		0.000132	½		0.1726	
¾		0.0339	¾		0.0497	¾		0.0109	¾		0.000345	¾		0.000111	¾		0.1452	
11 D	=	*0.0285*	*13 D*	=	*0.0418*	*14 D*	=	*0.00916*	*19 D*	=	*0.000290*	*23 D*	=	*0.0000933*	*13 D*	=	*0.1221*	= *8192 C*
¼		0.0240	¼		0.0351	¼		0.00770	¼		0.000244	¼		0.0000785	¼		0.1026	
½		0.0202	½		0.0295	½		0.00648	½		0.000205	½		0.0000660	½		0.0863	
¾		0.0170	¾		0.0248	¾		0.00545	¾		0.000173	¾		0.0000555	¾		0.0726	
12 D	=	*0.0143*	*14 D*	=	*0.0209*	*15 D*	=	*0.00458*	*20 D*	=	*0.000145*	*24 D*	=	*0.0000467*	*14 D*	=	*0.0610*	= *16384 C*
¼		0.0120	¼		0.0176	¼		0.00385	¼		0.000122	¼		0.0000392	¼		0.0513	
½		0.0101	½		0.0148	½		0.00324	½		0.000103	½		0.0000330	½		0.0432	
¾		0.0085	¾		0.0124	¾		0.00272	¾		0.000086	¾		0.0000277	¾		0.0363	

[4]Periodic data are based upon International Atomic Weights, September, 1951 *(92)*. The natural variation for compounds containing isotopes of sulfur is ±0.003. Copyright, 1953, by W. Franklin Dove.

tarsal, antennal, or internal. In some forms, as in frogs or fishes, the reaction or response may be observed only as muscular twitching or as a change in the membranes observed under the microscope. According to data available from numerous sources the taste response, and chemoreception in general, regardless of the species or the part of the body responding to stimuli, is amenable to scaling in D-Units.

The D-Unit, representing a magnitude of 100%, is wide enough for the first scanning of population groups both for thresholds of perception of a difference and of recognition of the stimulus. Single D-Unit values slightly above recognition levels may serve for identification levels, and for diagnosis of neural disturbances or of physiological or genetic differences.

For some taste-stimulating chemicals in solution the D-Unit may be found too wide with classes too few. The unit can then be reduced conveniently to any of its fractions, e.g., ½ D's or ¼ D's, without breaking away from the basic D-Unit series. By adjusting class interval, first, to the breadth or range of the chemical stimulus associated with successive dilutions, and, second, to the extent of the taste responses, one may be assured of any ideal number of class intervals for any soluble or miscible substance. At the same time the same data retain the accumulative and comparative advantages provided by basic attachment to the master gustometric scale of D-Units.

The master D-Unit scale is set up in Figure 1 to cover a range of 33 D-Units, contained in 11 log cycles; this range goes from the most concentrated foods (i.e. from a concentration of 100.0 parts into 0.4 parts) down to very dilute solutions of chemicals (i.e. down to a dilution of $\frac{1}{2^{24}}$). The scale for each chemical has its own independent and different range upon the master scale; nevertheless each chemical, from saturation point to taste extinction, extends on the scale approximately the same length: i.e., 15 D-Units, or within 5 log cycles. The most dilute of the five primary taste solutions in Figure 1 is a solution of quinine sulfate, where 24 D ($\frac{1}{2^{24}}$) represents a dilution rate of 1:21,000,000. Yet even this low dilution level can be diluted further, at will, since there is no end to the dilution scale.

The gustometric scale has been calculated for all 5 primary taste substances—sucrose, sodium chloride, tartaric acid, phenylthiocarbamide, quinine sulfate—in terms of ¼ log D-Unit values (Table 2). This guide table of values, spanning the range of dilutions from saturation to subliminal thresholds, embraces for all disciplines the significant area for graded taste stimulus-response relationships.

The Micro taste scales. The Micro taste scales represent class intervals of ¼ and ½ log size of the D-Units, i.e., 19 to 41% as increments. These micro intervals therefore approximate in magnitude the size of JND's as reported in Table 1, scales 1 and 3. As will be shown, these Micro scales are suitable for taste-matching and for the measurement of taste levels in establishing specifications and standards for quality development and control.

In order to determine this value of ¼ log D-Unit size as the proper taste focus for food specifications, a method was devised whereby the test item was bracketed from both higher and lower levels with successively smaller fractions of the D-Units until the point was reached where a finer fractionation of the D-Unit would not improve the accuracy of the test. An example of this procedure is shown in Table 3.

In column 1 of Table 3, full-sized D-Units bracket the sweetness level of a protein supplement beverage between 2 D and 4 D. Even though the results were consistent in all 5 repeated trials, the difference of 2 D-Units (from 2 D to 4 D) is too wide for practical use. In 3 repeat tests (15 trials), using smaller (½ log) D-Units, the sweetness level was bracketed in all 15 trials between 2½ D and 3½ D. This result is consistent and is brought within closer boundaries to the actual value, but its consistency indicates that even finer bracketing may be used successfully. When a finer bracketing (¼ log D-Units) was used, a consistent value of approximately 3⅛ D-Units or 3.9% sucrose-equivalence was secured. However, the fact that the taster recorded 10 of the 30 test items 3 D, and 5 of the 30 test items 3¼ D, indicates that the limit of fractionation for this product had probably been reached—at a scale interval or span of ¼ log D-Units. It has been noticed that the ¼ D-Unit scale interval serves to measure the breadth of the taste peak in different food substances, and the individual differences in taste focusing ability between tasters.

Bracketing the taste-equivalence of foods at ¼ D-Units also represents an acceptable tolerance of variation in taste control for use in formulations and specifications.

TABLE 3

Establishment by taste matching of the magnitude of the interval for Micro taste scales (for food specifications and formulations)

Example: A protein supplement reconstituted as a beverage, tested during 5 consecutive days (in 9 trials with 5 to 10 repetitions per trial).

Micro scale in ¼-log D-Units	5 tests by D-Unit intervals	15 tests by ½ D-Unit intervals			30 tests by ¼ D-Unit intervals					Sucrose equivalence to D-Units
1 D	5/0[e]									17.12
¼										14.39
½										12.10
¾										10.18
2 D	5/0	5/0	5/0	5/0			5/0			8.56
¼								5/0		7.20
½		5/0	5/0	5/0	5/0	5/0	5/0	10/0		6.05
¾					5/0		5/0	10/0	5/0	5.09
3 D	[4/1][f]	[4/1]	[1/4]	[4/1]	5/0	3/2	[1/4]	[6/4]	5/0	4.28
¼					2/3	1/4	0/5	2/3	0/5	3.60
½		0/5	0/5	0/5	1/4			0/10	0/5	3.03
¾					0/5	0/5				2.54
4 D	0/5	0/5	0/5	0/5						2.14
¼										1.80
½										1.51
¾										1.27
5 D	0/5									1.07
¼										.90
½										.76
¾										.64
6 D										.53

[e] The numerator refers to the number of trials in which the scaling solution was sweeter than the unknown test item in paired comparisons. The denominator refers to the number of trials in which the scaling solution was less sweet than the unknown test item.

[f] Brackets indicate the value nearest equivalence between scaling solution and the unknown test item.

This statement is based upon taste measurements made on many different classes of foods and beverages used in hospital feeding.

Attention is drawn especially to 4 foods as representative examples of the use of the Micro D-Unit scale, namely, canned applesauce, as a relatively homogenous but processed food; a protein supplement, representing a highly concentrated food requiring reconstitution before it can be consumed as a beverage; and fresh liquid milk, representing an unaltered natural food when whole, and the same food deprived of one constituent (fat) when tested as skim milk.

The sucrose-equivalence in Micro D-Units for the sweetest and least sweet of 10 brands of canned applesauce was 6.05%, and 3.60% respectively. The sucrose-equivalence of the sweetest and least sweet of 10 formulations of protein supplements was

3.90%, and 0.0%, respectively (Table 4). Skim milk was 1.51% and whole milk was 1.96% sucrose-equivalent (Tables 4 and 5).

The sweetness of the 10 protein supplements is definitely below the sweetness of applesauce, as processed and canned today with sugar added. Yet the sweetest protein supplement was rated "too sweet" by the consumer panel, while the least sweet brand of applesauce, though rating approximately the same as the sweetest protein supplement in sucrose-equivalence on the gustometric scale, was considered by the consumer panel as "too bland."

This relative response to sweetness level in different foods serves to demonstrate the tie-in between affective responses and the standardized D-Unit gustometric scale. By

TABLE 4

Sucrose equivalence of protein supplements in Micro D-Units

Micro scale in ¼ log D-Units	Standards for comparison		Ten leading brands of protein supplements, reconstituted into milk-like beverages[g]										Sucrose equivalence to D-Units
	Fresh liquid skim milk	Fresh liquid whole milk	A	B	C	D	E	F	G	H	I	J	
													%
1 D													17.12
¼													14.39
½													12.10
¾						1/0[j]							10.18
2 D						[1/1]							8.56
¼						0/1							7.20
½													6.05
¾									4/0	5/0			5.09
3 D				5/0					3/2	5/0			4.28
¼				5/0					1/3	1/3		5/0	3.60
½				[1/3]				5/0	0/5	1/4	4/0	5/0	3.03
¾				0/5				5/0	0/4	0/4	4/0	[2/2]	2.54
4 D	5/0[h]	5/0		0/5	5/0			0/4	0/1		[2/3]	0/5	2.14
¼	4/1	0/5			5/0			0/4			0/4	0/5	1.80
½	[2/3[h]]	0/5			[2/3]			0/4			0/4		1.51
¾	1/4				0/5								1.27
5 D	0/5				0/5								1.07
¼													.90
½													.76
¾													.64
6 D													.53
			Nil[i]				Nil						

[g] Data in this table, as well as in Tables 3 and 5, were secured as part of a comparative study of protein supplements as to consumer acceptance, supported by grants from Mr. Howard Bishop, Summit, New Jersey, to the University of Illinois College of Medicine.

[h] The numerator = number of times the D-Unit scale solution is considered *sweeter* than the test item; the denominator = number of times the scale solution is considered *not as sweet* as the test item.

[i] Since the average sucrose taste threshold is approximately 6 D, a record of "Nil" is given to any item in which the quality of sweetness cannot be recognized by taste.

[j] Item D was given only a spot taste test; the high medicinal flavor made further tests of little value until the dominant medicinal taste is nullified.

[k] The brackets indicate the approximated value for sucrose equivalence of each item.

TABLE 5

Sucrose equivalence of reconstituted protein supplements. An example of the shift in taste level of a single protein supplement, reconstituted at different levels of concentration, and in different solvents (e.g. water, skim milk, fresh milk)

Micro scale in ¼ log D-Units	Sucrose taste equivalence of protein supplements reconstituted in water to equal milk of 3.5% protein, or its multiple					Sucrose taste equivalence of liquid milk used as reference standard		Effect of solvent on taste equivalence when reconstituted			Sucrose equivalence in D-Units
	1 MPLE[1]	2 MPLE	3 MPLE	4 MPLE	5+ MPLE	Fresh skim	Fresh whole	In water 2 MPLE	In fresh skim milk 2 MPLE	In fresh whole milk 2 MPLE	
											%
1 D											17.12
¼											14.39
½											12.10
¾				5/0						5/0	10.18
2 D			5/0	5/0					5/0	5/0	8.56
¼		5/0	5/0	1/4					5/0	5/0	7.20
½		10/0	5/0	0/5					4/1	0/5	6.05
¾		10/0	1/4					5/0	1/4	0/5	5.09
3 D	5/0	6/4	0/5					5/0	0/5		4.28
¼	5/0	2/3						0/5			3.60
½	4/1	0/10						0/5			3.03
¾	1/4										2.54
4 D	0/5					5/0	5/0				2.14
¼						4/1	0/5				1.80
½						2/3	0/5				1.51
¾						1/4					1.27
5 D						0/5					1.07
¼											.90
½											.76
¾											.64
6 D											.53

[1]MPLE = Milk Protein Level Equivalence: a convenient method of referring to the concentration level of any reconstituted food used in high protein feeding, when the dehydrated powder can be added conveniently to water, milk, fruit juice, or other solvent, to provide a liquid diet higher in protein value than is usually obtainable from natural liquid foods. 4 MPLE in water contains 14% protein; 4 MPLE in milk contains 17.5% protein.

this method subjective responses can be anchored to permanent fixed points on a standard reference scale: National and international preferences and prejudices can be measured and compared by such a taste meter.

For example, if the concentrated protein supplements are to be used like milk beverages, we may wish to reformulate them according to the sweetness level of some well-known standard, like whole fresh milk. To make such corrections for sweetness, we would *reduce* the sweetness level of protein supplement B, and *increase* the sweetness level of C. However, if the protein supplement is to be *added* to fluid milk rather

than to water as solvent, or if it is to be used as a high protein supplement and is made up to 3 or 4 times the milk protein level (3 MPLE or 4 MPLE), then the B protein supplement might be excessively sweet, while the C might not require any additional sweetening.

On the other hand, suppose these protein supplements were to be shipped abroad as bulk concentrates to foreign hospitals, as in India, where more sugar or sweetening is commonly added to milk and to milk products *(32)*. In that case the higher sweetness levels might be desired. The record on the permanent fixed sucrose log scale would provide the necessary data for international comparisons and specifications.

When taste levels for any food are not in agreement with good nutrition, the D-Unit values serve as bench marks for the measurement of subsequent progress in the production of therapeutic foods and in medical nutrition education.

The gustometric measure of taste level could also have bearing on Food and Drug regulations. As for applesauce, the question can be raised: "What is the definition of a sweet, or a 'tart' applesauce?" or "When is sugar added?" The answer cannot be secured in terms of degrees of Brix, or of refractometer readings. It is necessary, first, to establish the optimum range by taste level marked off on the standard gustometric scale. When these taste levels are established in the specifications, first according to consumer acceptance, instrumentation may then take over and provide some of the subsequent control upon the new formulation through the use of refractometer, hydrometer, degrees Brix, pH meter, or other physical-chemical-mechanical device for quality control.

In the modern production of foodstuffs, the temptation has been great to bypass the 'too variable and unpredictable human responses,' and to go directly to instrument measurements for quality control. And yet these variable human responses can be brought within the range of prediction and control by means of statistical organization of responses on a universal standard of reference.

There is no instrument that can measure sweet, saline, sour, or bitter as such in the ordinary foods of the day. Only in specific and restricted instances with relatively simple items can this be accomplished. And yet these measures on the foods of commerce represent the taste levels needed most by the food engineer. The accentuation of natural taste may at times be considered illegal *(46)*, and the individual judgment of flavor or the consumer group opinion may be questioned. Taste levels in D-Units on the gustometric scale provide that standard information needed for specifications and regulations in taste quality control.

It will always be necessary to transpose the subjective taste (sensory) responses into the standard reference D-Unit values, before they can be in turn transmitted to the control of instruments.

The Ultramicro taste scales. The Ultramicro taste scales are among the finest subdivisions of the D-Units to which any organism may respond. For man they include the log fractions from $\frac{1}{8}$ to $\frac{1}{64}$, and smaller if necessary. At $\frac{1}{64}$, the D-Unit interval is reduced to an increment of 1.0889%, which is finer than taste discriminatory ability usually to be encountered, and equal to the difference in magnitude of stimulus required to elicit a response in the finest of sensitivities.

An example of the use of the Ultramicro taste scales in establishing the taste difference threshold for sucrose is shown in Table 6.

The test presented in Table 6 was set up to bracket (above and below) the $3\frac{1}{4}$ D-Unit level. The $3\frac{1}{4}$ D-Unit level is in the mid-range, and is equal to 3.60% sucrose solution. Out of 5 complete tests, the difference thresholds were as follows: 2.2%; 4.4%; $<$ 1.1%; 2.2%; and 4.4%. Even though the subject had a threshold of recognition for sucrose of 6 D (0.53%), which is average for population tests, the results of these discrimination sucrose tests are much lower than any yet reported for sucrose. Calculations from published data in Table 1 show previous low JND's of 16.3% and 28.6% for sucrose at the same level of sucrose concentration.

These low Weber fractions of 0.011 to 0.044 secured in these tests by successively smaller fractions of D-Units (Table 6) indicate that taste discrimination may not be as crude as the 0.30 average currently reported for it, but may rank equally with some of the finest discriminatory abilities, e.g., 0.01 for vision, 0.025 for perception of weight differences, and 0.05 for pressure on the skin *(58)*.

In order that difference-thresholds possess meaning, all discrimination tests and difference thresholds should be measured in relation to some universal standard of ref-

TABLE 6

Taste discrimination tests on the Ultramicro D-Unit taste scale for the Just Noticeable Difference in sweetness of sucrose in solution

Order of testing by pairs of s with d	Test sample s, sinistral member of the pair							Actual differences in percent sucrose between sample s and sample d			Test sample d, dextral member of the pair						
	Taste records in 5 repeated trials					D-Unit values in $\frac{1}{64}$ logs	Grams of sucrose per liter of solution	$\frac{s-d}{s}\cdot 100$	Δ in $\frac{1}{64}$ths	$\frac{s-d}{d}\cdot 100$	Grams of sucrose per liter of solution	D-Unit values in $\frac{1}{64}$ logs	Taste records in 5 repeated trials				
	Trial 5	Trial 4	Trial 3	Trial 2	Trial 1								Trial 1	Trial 2	Trial 3	Trial 4	Trial 5
							g.	%		%	g.						
Pair 1	$+_1$[m]	$+_2$[n]	$+_2$	$+_1$	$+_2$	3 D	42.79	29.3	32	41.4	30.26	$3^{32}/_{64}$ D	−	−	−	−	−
Pair 2	$+_1$	$+_1$	$+_1$	$+_1$	$+_1$	$3^{2}/_{64}$	41.87	26.2	28	35.4	30.92	$3^{30}/_{64}$	−	−	−	−	−
Pair 3	$+_2$	$+_1$	$+_2$	$+_1$	$+_2$	$3^{4}/_{64}$	40.97	22.9	24	29.7	31.59	$3^{28}/_{64}$	−	−	−	−	−
Pair 4	$+_1$	$+_1$	$+_2$	$+_1$	$+_1$	$3^{6}/_{64}$	40.10	19.5	20	24.2	32.29	$3^{26}/_{64}$	−	−	−	−	−
Pair 5	$+_2$	$+_1$	$+_2$	$+_2$	$+_2$	$3^{8}/_{64}$	39.24	15.9	16	18.9	32.99	$3^{24}/_{64}$	−	−	−	−	−
Pair 6	$+_1$	$+_2$	$+_2$	$+_2$	$+_1$	$3^{10}/_{64}$	38.40	12.2	12	13.9	33.72	$3^{22}/_{64}$	−	−	−	−	−
Pair 7	$+_2$	$+_2$	$+_2$	$+_1$	$+_1$	$3^{12}/_{64}$	37.57	8.3	8	9.1	34.45	$3^{20}/_{64}$	−	−	−	−	−
Pair 8	$+_2$	$+_1$	$+_2$	$+_1$	$+_2$	$3^{13}/_{64}$	37.17	6.3	6	6.7	34.83	$3^{19}/_{64}$	−	−	−	−	−
Pair 9	$-_1$	$+_2$	$+_2$	$-_1$	$+_1$	$3^{14}/_{64}$	36.77	4.2	4	4.4	35.21	$3^{18}/_{64}$	−	+	−	−	+
Pair 10	$+_2$	$-_1$	$+_2$	$-_1$	$-_1$	$3^{15}/_{64}$	36.37	2.1	2	2.2	35.59	$3^{17}/_{64}$	+	+	−	+	−
Pair 11	$-_2$	$+_1$	$+_2$	$+_1$	$-_2$	$3^{15}/_{64}$	36.37	1.1	1	1.1	35.98	$3^{16}/_{64}$	+	−	−	−	+
Pair 12	$-_2$	$+_1$	$+_2$	$+_1$	$-_2$	$3^{16}/_{64}$	35.98	1.1	1	1.1	35.59	$3^{17}/_{64}$	−	+	−	−	+

[m] The + sign indicates the member of the *s d* pair considered sweeter, while the − sign indicates the member of the pair considered less sweet.
[n] The sub-figure below the + or − sign represents the *presentation* order of the two members of the *s d* pair: 1 = member tasted first, 2 = member tasted second.

erence as represented by the D-Unit scale. Furthermore, by means of this bracketing device used in the discrimination test, the relation between stimulus and response may be studied over its entire range without restriction to any preconceived hypothesis of the psychophysical nature of the stimulus-response relationship.

Ultramicro taste scales have many potential uses as yet not fully explored: as spanning devices for the measurement of difference thresholds; as bench marks for the record of relative discriminatory ability in various subjects, normal, atypical, or chronically ill; for taste-matching food with chemical, or food with food; for bracketing an unknown fine taste on the chemical scale; for establishing taste and odor levels in water and beverages; and for measuring excessive or abnormal sensitiveness to taste stimuli on a universal standard in the diagnosis of suspected metabolic, psychiatric, and psychosomatic disturbances.

DISCUSSION

The D-Unit scale as a taste-measuring device in gustometrics. By definition, the gustometric D-Unit scale is a mathematical scaling device, adjusted to taste stimuli and responses, so as to provide a standard reference and an orderly arrangement required for the recording and the analysis of perception, recognition, acceptance, and rejection of saporous substances by the organism.

According to this definition, the D-Unit scale is neither a simple stimulus scale, a response scale, nor a physical scale *per se*. And yet by virtue of its mathematical construction (halving and finer fractioning) and its physical-chemical features (dilution or concentration), the D-Unit scale includes characteristics of all 3 classes of scales, in addition to the characteristics uniquely its own. For instance, in the successive dilutions and concentrations of chemicals or foods, the D-Units appear as, and are used as, different levels of stimuli. At these increasing concentration levels the stimuli are expected to evoke increasing (more or stronger) taste responses. But the D-Units are mere mathematical increments, or at best physical divisions of solutions or of food substance of varying degrees of homogeneity, and are not constructed as measured or graded scales of increasing or decreasing taste stimuli. A true stimulus scale would be constructed as a uniform graded series of a single mode of taste-evoking impulses. But even with any one of the so-called primary taste-stimulating chemicals in solution, a simple stimulus scale is seldom produced by dilution units. When considered as stimulus scales, primary solutions are usually taken from a mid-section of the dilution range in order to evade confusion of stimuli to be encountered at high concentration levels *(55)*. Furthermore dilution scales are usually multi-modal in the character of responses elicited from the taster *(29, 77)*, a feature widely recognized also in striking form with the different taste responses to PTC and other chemicals in solution *(9)*.

Pure sucrose or pure sodium chloride, etc., dissolved in pure tasteless and odorless water, elicits responses with different degrees of pleasantness and unpleasantness at different concentrations *(29, 69)*. Acid and salt solutions may be pleasant at dilute levels and unpleasant and bitter at high concentrations *(77, 89)*. Even *bitter* has its pleasant moments at very dilute levels, occasionally and when combined or associated with different tastes *(23, 29, 59)*.

Other changes also take place with dilution and concentration, i.e., changes in consistency, viscosity, body, specific gravity, texture, color, in

degree of ionization, and in pH. These variants of the stimuli at different levels of dilution help to produce the multi-modal taste response. The pattern and nature and degree of response for the same sapor may even vacillate back and forth with different degrees of concentration and dilution. For these reasons D-Unit scales may even hold characteristics usually confined to response scales. In taste-matching, response and stimulus scales (with 3 classes, e.g. +, =, −) actually become interchangeable.

Stimulus scales (as a series of increasing weights) are usually discrete and separate from response scales. In taste, too, response scales are developed in many instances separately from the stimuli they are to measure. These response scales include, for taste, degrees of like or dislike, of pleasantness or unpleasantness, of acceptance or rejection, hedonic tone, affective responses, and taste intensity.

Subjective responses have been extensively treated in terms of preference *(94)*, scaled in terms of intensity as gusts *(5, 6)*, and statistically organized in terms of laws of comparative judgment, rank order, and prediction of choice *(86)*. The D-Unit scale device may form, under specified restricted conditions, a true scaling of stimuli or of responses or of physical features, but it is not always any one of these in simple form. The extent even to which it may be used for any one of these features remains yet to be determined.

There is another distinction to be made between the D-Units as stimulus scale and the subjective response scales — usually encountered in taste-matching. Take as examples the 2 items applesauce and a protein supplement reconstituted into a milk-like beverage; both items may have a "sweetness" rating equivalent to 3% sucrose solution on the D-Unit scale. However, this rating does not imply an equal sucrose content of 3% in the 2 different foods. The subjective response is recorded merely in a chemical sucrose scale value, and may or may not be (and usually is not) comparable to the specified sucrose concentration. Foods may contain lactose, levulose, or saccharine, and their combined subjective effect, by virtue of the test for differences, is combined into a sucrose-taste-equivalence. Such a sucrose-taste-equivalence as 3% in applesauce can be arrived at or duplicated by any number of permutations and combinations of sugars, acids, and astringent factors *(18)*. Even physical factors, like the size of crystals or homogenization, alter the perception of sweetness.

A knowledge of the competitive and compensatory and complementary action between acids, sugars, salts, and other food constituents is basic to food acceptance *(30)*, and yet the resulting taste levels must and can be translated on to the D-Unit scale. In food engineering practices, salt is often required at different levels in different kinds of foods, even for different kinds of candies, to reduce the sourness of acids or to increase the sweetness of sugars *(21)*.

The value of the standard taste level scale in D-Units is the universal comprehension and duplication of the 3% sucrose-taste-equivalence, or any other level of any taste wherever needed. And the need arises when any D-Unit value reprsents optimum consumer acceptance or consumer need, and when deviations from that D-Unit value represent rejection of the food, or result in deficiency status of the consumer.

The question can be raised: Do D-Units provide a measure of taste in subjective taste units? This question may be answered as follows: Subjective taste units or increments of taste discrimination vary from individual to individual, and from condition to condition. Nevertheless, psychological taste scales have been developed in terms of gusts as a subjective measure of taste intensity *(5, 6)*, and demonstrate interesting possibilities in use. However, the psychological taste scale reaches a 100% ceiling beyond which it cannot go, leaving some foods of high taste intensity—like lemons, etc.—unscalable *(6)*. On the other hand, any subjective taste unit can be localized and described (in amplitude, range, or intensity, etc.) at any specified locus on the D-Unit scale. The D-Unit scale as a gustometer provides impersonal taste meter markings as permanent limitless standards of reference. Its value lies in the subsequent use to be made of the standardized information, in the same manner that information secured with the mercury thermometer acquires value only to the extent that the information can be and is used.

The mathematical and physical features unique to the D-Unit scale extend beyond stimulus, response, or physical scales as such, and are imbedded in the structure of the progression series originating at a fixed point or universal standard of reference, i.e., at one concentration, the point between the dilution and the concentration series.

In psychometrics an absolute zero has been considered desirable for psychological scales, if attainable, but not considered essential *(40)*. For ratio scales an absolute zero is always implied even though the zero value on some scales (e.g., Absolute Temperature) may be unattainable *(85)*. In the measurement of taste of foods and in the measurement of the ability of subjects to taste saporous substances, a central point of origin possesses greater significance and is attainable. For instance, the process of eating foods is a process of *gradual dilution*, abetted by comminution, salivation, rate of acceptance, mixture with other foods, and alternate imbibition. So too in the protection against poisons—the nibble or the sip provides also the *gradual increased concentration* necessary for protection from toxic substances while approaching the threshold of acceptance or rejection. The central fixed point of reference in the midst of this universe of tastable chemical compounds and natural foods (and their would-be substitutes or counterfeits, the toxicants and poisons) have a definite ontogenetic and phylogenetic bearing upon survival and the nature of the surviving type. For such, the D-Unit scale provides the desired central standard anchorage for its origin, and also the frame of reference for the unified dilution scale required in a science of gustometrics.

Comparative gustometrics in D-Units. In comparative gustometrics, solubility for chemicals in solution has a parallel in the shift in ratios of ingredients in solid foods. This fact takes on current importance to both producer and consumer as food processing comes, more and more, to include straining, chopping, homogenizing, pulverizing, centrifuging, rolling, separating, refining, and remixing, with the necessity for increased attention to the different effects of these processes upon the taste acceptance of the final product.

Dehydration and concentration of foods has come to be encouraged because of the decrease in packaging and shipping costs. The subsequent necessity for reconstituting the concentrate raises taste problems which parallel the taste problems for chemicals in solution. Of the starches, fats, emulsions, gels, casein in milk, some are pseudo-solutions and colloidal in nature. Yet these constituents go through the diluting process (or reconstitution) as though they were true solutions.

Although the parallel between liquid solutions and solid solutions indicates a common use for the D-Unit scale, irregularities in action of these solid solutions demonstrate, as with true solutions, that they do not provide taste stimuli in a simple graded series. The salts, acids, and bases may dissociate and require buffers at certain levels of concentration or dilution. During reconstitution the starches or yeasts may settle out, the fat may rise, while the lactalbumins may overflow in foam. Stabilizers, neutralizers, emulsifiers, and wetting agents may have to be added to take effect at some levels, and not at other levels, of dilution (reconstitution) in order to maintain taste acceptance. These variables, concomitant with stage and level of concentration and dilution, represent examples of a few of the disruptions to the concept of a simple taste stimulus scale associated with dilution as such.

Obviously the D-Units as loci on a metered scale are relatively free from these distractions and are able to serve as standard bench marks for the recording of any one of these and other changes which may affect taste at each different level of concentration, reconstitution, dilution, or mixing of any food product.

The centrally controlled successive dilution method has other advantages besides its parallelism with the processes inherent in successive concentration and reconstitution of foods. Infinitesimal quantities of chemicals and tastable substances, too small to weigh on an analytical chemical balance, can be measured by gustatory and olfactory responses of the subject. By dilution procedures the usual chemical laboratory accuracy of $\pm$ 0.0001 for weight can be exceeded or extended considerably. As a result the measurement of the finer shades and variations in taste perception may be improved as diagnostic signs in psychiatry, pathology, and clinical medicine.

The gustometric scale as drawn in Figure 1 extends from 2^{0} to $\frac{1}{2}^{24}$, and covers a range from highly dehydrated food items reduced to 0.4% to dilutions of 6/100,000,000. Since the progression series is without end, these extremes do not represent limits. Also, since all scale units can be subdivided endlessly, some predictions can be made on the use of the scale in gustometry.

The universal gustometric scale (Figure 1) provides the common denominator for which we sought to underline many separate floating islands of taste research. To mention a few, together with selected references, the D-Unit scale can include within its framework the following diverse interests:

1. Individual differences in responses to the primary tastes of sweet, salty, sour, and bitter *(9, 33, 78, 79)*.
2. Use of taste response in neurological and anatomical studies *(64, 68, 69)*.
3. Relation between concentration and affective responses, hedonic tone, and other subjective responses *(56, 94)*.

4. Taste type and taste levels used for diagnostic purposes in injury and in disease *(12, 43)*.
5. Taste levels for hypergeusesthesia *(76)*, ageusia *(70)*, cacogeusia *(84)*, after-tastes and ante-tastes, taste aura *(13, 82)*, etc.
6. Hyper- and hypo-sensitivity as thresholds of perception and recognition, as in diabetes *(92)*, lead poisoning *(88)*, effect of pregnancy *(70)*, and metabolic disturbances of the thyroid *(43)*.
7. Degrees for taste acuity for taste judges and chefs *(17, 26)*.
8. Degrees for taste-discriminatory ability *(7, 49, 51, 54)* and taste-matching *(6)*.
9. Permanent bench marks for taste levels in formulations, recipes, and food specifications (Tables 4 and 5).
10. Analysis of taste and appetite or hunger *(38, 50)* in specific diseases, such as tuberculosis, and chronic illness *(37)*.

In addition to taste measures on the subject or patient, the same scale includes taste measurements used in food technology, i.e.:

11. Chemistry of taste *(22, 58)*.
12. Taste levels for all natural foods and their genetic relatives, as well as for those which have been dehydrated, processed, condensed, concentrated, reduced, or diluted.
13. Intimately associated with the measurement of taste problems in producing *(39)*, preparing, and processing foods *(23, 26)*, the taste measurements for all of these foods when reconstituted or prepared for the consumer.

The analysis of the literature in each one of these 13 areas in which taste measurements are possible or have been required, and the suggested uses which could be made of the D-Unit device in each of these areas, go beyond the purpose of this article. Needless to say, however, the research in each area has been quite largely detached and isolated by the lack of a standard frame of reference for the correlation of stimuli with responses. This reference standard is supplied by the D-Unit device.

The universal adaptibility of the D-Unit scale. Aside from the wide applicability inherent in the D-Unit scale previously noted, there are characteristics in this device which are uniquely universal. Although some of these characteristics have already been referred to incidentally, they merit summarization, which follows:

1. The scales lack ceilings, and are therefore capable of metering any level of concentration or dilution desired.
2. A universal taste focus is provided by a limitless fractionation of scale segments.
3. Units may be treated as of an arithmetical or logarithmic series, or by any formula which may fit the data.
4. A duality exists between dilution and concentration, whereby either dilution or concentration is a reverse image of the other at any level, and either may be substituted for the other when freed from the fixed point of origin.
5. An identity exists between the scale for chemicals in solution and the scale for the concentration and dilution of natural foods.
6. The gustometric scale should be potentially capable of metering all taste responses from taste stimuli received from any saporous substance, by any organism, anywhere. This same D-Unit scale, although drawn up here in Figure 1 for man, has the flexibility and extensionality to encompass the taste responses in all food-getting organisms capable of responding to such stimuli.

All of these universal features are incorporated without detraction from and, in fact, to the advantage of comparative taste analysis.

These conclusions in regard to the universality of the gustometric scaling concept do not impinge upon taste-testing procedures used, nor upon the use of psychological taste scales, nor upon the physico-chemical, psychophysical, or clinical and medical concepts of taste; but may assist in their organization, development, and interpretation. The relationships are the subject of continuing investigation.

SUMMARY AND CONCLUSIONS

The present report deals with the development of a universally adaptable gustometric scaling device, where now there are many scaling methods and a plethora of taste scales, but without a unifying principle.

An analysis of past and current taste scales used in numerous disciplines reveals the nature of the major deficiencies, and explains the need for an underlying standard of reference in this science.

In the light of these obvious past difficulties, and on the basis of experience with present need and future potential demands in taste acceptance research, a universal gustometer or taste meter has been devised. The taste meter is not alone a stimulus scale, a response scale, or a physical scale, but is rather a mathematical progression scale, subdivided according to the magnitude necessary to record the taste changes that occur when foods or soluble chemicals are diluted or concentrated. The taste meter serves as a universal and permanent frame of reference for stimuli, responses, and physical changes concurrent with dilutions and concentrations.

The gustometric scale consists of a centrally anchored progression series of dilution units adjustable in scale interval to the magnitude of the change in stimulus required to elicit specific measurable taste responses.

Other characteristics of the taste meter include the following, as described in the text with examples:

1. The taste meter is marked off from a central point of one molar solution for soluble chemicals, and of one part by weight or volume for natural, processed, or synthetic foods.

2. This fixed point of origin, one concentration, is at the center of the universe of tastable substances and of responses by tasting organisms.

3. The mathematical formula which serves best the myriad requirements for taste is the exponential formula of $\frac{1}{2}^n$ for dilutions, and 2^n for concentrations. The exponent for dilutions represents the number of D-Units, and provides for rapid calculation, comparison, and reproduction of the same unit level of taste solutions anywhere, any time, in any discipline.

4. The same series of D-Units serves as a progression series for both dilutions and concentrations, since:

(a) one concentration represents the central point common to both series;

(b) the same formal statistical order for the dilution series forms a reverse series for the concentrations, and the 2 series are therefore interchangeable;

(c) the scales have no terminal points, have no ceilings, do not end;

(d) the same dual scale measures both chemicals and foods, natural as well as processed foods—be they concentrated, dehydrated, diluted, mixed, or reconstituted; and should therefore be capable of measuring any saporous substance to which there is a graded response.

5. The taste scale, subdivided according to the magnitude of the responses secured from tests over the dilution range, provides a universal taste focus of many taste situations, responses, and problems. Universal taste focus is secured by successive fractionation of the D-Units. Three useful scale intervals are described, with examples:

(a) The Macro D-Units of 1 D (increments of 100%) are suitable to the scaling of taste thresholds, serve as loci for records of affective responses, and as levels for establishment of relative acuity of food judges and the extent of pathological change in taste or presence of genetic differences in taste ability.

(b) The Micro D-Units of ¼ D (increments of 18.9207%) serve as bench marks for taste matching, for food specifications, formulation, and establishment of levels for reconstitution of dehydrated foods.

(c) The Ultramicro D-Units, fractionated to $\frac{1}{64}$ D (increments of 1.0889%), or smaller if necessary, mark off the degrees of fine taste discrimination and establish taste difference thresholds.

6. Since the D-Unit scale is endless, it is capable of extending beyond (and therefore includes) the entire range of any taste stimulus continuum.

7. Each of the 5 primary taste solutions (sucrose, sodium chloride, tartaric acid, phenylthiocarbamide, and quinine sulfate) is found to span 4 to 5 log cycles, regardless of their different positions on the master scale. The entire range of tastable substances on the master gustometric D-Unit scale falls generally within the span of 10 to 11 log cycles.

8. The D-Unit scale has the multiple advantage provided by adaptability to mathematical treatment as a logarithmic, arithmetical, numerical, or other progression series.

9. The taste response features, measured by numerous investigators, on different scales in many different disciplines, appear to be measurable to advantage on this one D-Unit scale.

10. Since the D-Unit scale is adaptable to the measurement of graded taste responses to a graded series of taste stimuli, on an endless scale, it should also be adaptable to the measurement of taste, smell, and the common chemical sense in insects and animals, as well as in man, regardless of the organ or structure responsive to the different stimuli.

A taste spectrum, calculated for all 5 primary taste substances and for natural or processed foods, with dilutions dispersed according to ¼ log D-Units, is presented in table form, for use by any discipline in focusing upon taste responses in any significant area between saturation and subliminal thresholds of perception of a difference in taste.

The gustometric scale is presented also in graphic form, together with test examples of its use in evaluating foods and subjects. These applications include assays of the primary taste thresholds, difference thresholds,

examples of taste-matching, and the establishment of taste levels for the proper reconstitution of protein supplements to serve as taste standards for food specifications. All of these features are marked off on the universal gustometric scale.

It is concluded, therefore, from a combined analysis of data available in numerous scientific reports in many different disciplines, and from critical test experiments by the writer, that D-Units of 100% magnitude, anchored to one molar solution and to food volume or weight as the central fixed point of origin, and forming a progression series of scales amenable to sub-fractioning (arithmetical or logarithmic) as parts to the whole, represent the common denominator and frame of reference best fitted to measure and unify the diverse phenomena to be met in a science of gustometrics.

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THE FAT-GLOBULE MEMBRANE OF NONHOMOGENIZED AND HOMOGENIZED MILK. I. THE ISOLATION AND AMINO ACID COMPOSITION OF THE FAT-MEMBRANE PROTEINS[a]

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More than 100 years ago, Ascherson *(1)* theorized that the fat particles in milk were surrounded and stabilized by a "haptogenic membrane" assumed to be formed by the condensation of albumin at the fat/plasma interface. Since that time numerous components of milk have been associated with the fat-globule membrane of nonhomogenized milk, namely: lacto-fibrin *(2)*, albumin *(3)*, a mucin-like protein *(29)*, serum proteins *(31)*, "haptein" *(10)*, "schaumstoff" *(25)*, casein *(30, 37)*, globulin-like proteins and phospholipids *(22, 23)* and a protein-phospholipid-glyceride complex *(12, 13, 26)*. A number of minor substances have been included also such as: flavoprotein or xanthine oxidase *(27)*, copper-containing proteins *(6)*, agglutinin *(28)*, carotenoids *(16)*, leucocytes *(24)*, phosphatase *(14, 26)*, and lipase *(17)*.

Interestingly, in most instances a protein has been identified as, or associated with, the fat-globule membrane of normal milk. In a few cases these proteins have been characterized principally through the application of Van Slyke's nitrogen distribution technique and selected elementary analyses *(10, 26, 30, 38)*. Only limited data concerning a few of the amino acids present in the fat-membrane protein have been recorded in the literature *(10, 26, 30, 38)*. In a preliminary report *(4)*, a comparison was made between the concentration of 13 amino acids in fat-globule membrane proteins prepared from nonhomogenized and homogenized milk. A more recent paper by Hare, Schwartz, and Weese *(9)* listed the concentration of 16 amino acids in the fat-globule membrane protein of nonhomogenized milk.

Although there are no data in the literature which show the effect of homogenization on the composition of the fat-membrane proteins of homogenized milk, there are indications that the nature of the fat/plasma interface is changed markedly by homogenization. Wiegner *(37)* has calculated that the amount of casein that could be adsorbed on the fat surface in nonhomogenized and homogenized milk is about 2 and 25%, respectively, of the total available casein. He assumed that casein was the sole substance adsorbed. Doan *(7)* believed that the decrease in the heat stability of homogenized milk resulted from an increase in the amount of casein adsorbed on the enlarged fat surface. Chambers *(5)* reported that the reduc-

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tion in curd tension accompanying homogenization resulted from an increase in the number of fat particles which served to weaken the curd matrix by providing an increased area upon which the plasma proteins become fixed. Weinstein and Trout *(36)* explained the increased susceptibility of homogenized milk to develop the solar-activated oxidized flavor on the basis that some of the oxidizable milk components were redispersed on homogenization and adsorbed on the newly formed surfaces. Moore and Trout *(19)* believed that the increase in the viscosity of milk homogenized at lower temperatures was due to an increase in the adsorption of proteins on the fat globule.

Webb *(34)* reported that homogenization of pasteurized milk was accompanied by a corresponding increase in the surface tension of milk as the homogenization pressure was increased and the degree of fat dispersion became greater. Trout, Halloran, and Gould *(33)* presented evidence to show that the foaming capacity of homogenized milk was increased by homogenization. King *(15)* stated that the amount of free fat (hydrophobic) was reduced by homogenization. These and other observations indicate that the newly formed fat surfaces, resulting from homogenization, were covered, in part, with proteins adsorbed from the milk plasma. Such a possibility was considered by Trout *(32)* who stated: "From the effects of homogenization on some of the physical properties of milk, it may be presumed that a film of protein materials is absorbed to the surfaces of the homogenized fat globules. Thus, the newly created fat globules are 'resurfaced.' The absorbed layer appears to be different from that on normal fat globules."

The present paper is the first report of a series of investigations concerning the nature of the fat-globule membrane obtained from nonhomogenized and homogenized milk and deals specifically with an isolation technique and a comparison of the amino acid concentrations in the 2 fat-globule membranes.

EXPERIMENTAL PROCEDURE

The milk used in this study was pooled herd milk which had been received and processed in the Michigan State College Creamery during the summer months. The average composition was 3.80% fat and 12.30% total solids.

Fat-globule membrane proteins were prepared according to the flow diagram shown in Figure 1. Forty gallons of pasteurized, nonhomogenized milk and 70 gal. of homogenized milk were separated to provide an adequate working supply of the fat-membrane proteins. The isolation procedure was based on a dilution and washing technique proposed by Palmer and his colleagues *(12, 13, 23, 26, 38)* in their classical studies with the fat-globule membrane. Their procedure for extracting the lipid materials from the proteinaceous materials of the fat-globule membrane consisted of refluxing the isoelectric, dialyzed fat membrane with successive portions of acetone, absolute ethanol, and purified ethyl ether. In the present study, the lipid materials were extracted, first, with cold ethanol-ether similar to the technique used by Hardy and Gardiner *(8)* to separate the lipids from blood proteins. This treatment was followed by extraction with 40°C. ether. The extraction technique was as follows:

(a) Three volumes of cold (0° to 3°C.) absolute ethanol were added to the cold (0° to 5°C.), dialyzed concentrate of fat-globule membrane materials. This mixture was agitated 10 min. by means of a mechanical stirrer and then filtered through a pleated Whatman No. 2 filter paper. The residue was washed 3 times with cold, purified ethyl ether.

(b) The precipitated protein material was added to 500 ml. of ethyl ether. The temperature of the mixture was adjusted to 40°C. and agitated for 15 min. The mixture

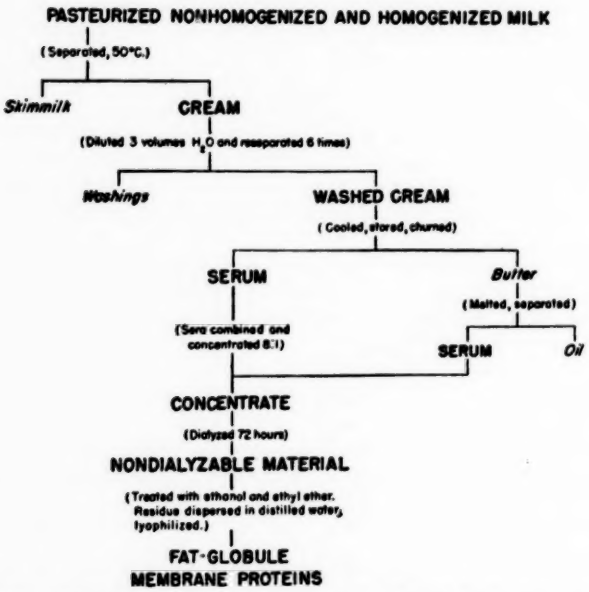

Figure 1. Flow sheet showing the procedures followed in the preparation of fat-globule membrane proteins.

was transferred to a separatory funnel to allow the precipitate to settle. The precipitate was drawn off and extracted 2 more times with lesser quantities of ether. The final separation of precipitated fat-membrane proteins and ether was accomplished by filtration through a No. 2 Whatman paper.

(c) The protein residue on the paper was washed several times with ether and then dispersed in a small amount of distilled water, lyophilized, and stored in the dry state at −20°C.

Nitrogen was determined according to the semimicro-Kjeldahl technique of Menefee and Overman *(18)*.

All of the amino acid determinations were made by the same standard microbiological techniques as outlined by Weinstein, Duncan, and Trout *(35)*.

RESULTS

Analytical results obtained from the microbiological assay of the fat-membrane proteins of nonhomogenized and homogenized milk are given in Table 1. The amino acid composition of milk, the minor-protein fraction, casein, lactalbumin, β-lactoglobulin, pseudoglobulin, and euglobulin are presented for comparison. Microbiological analyses for arginine, histidine, isoleucine leucine, methionine, phenylalanine, threonine and valine made on fat-membrane proteins from nonhomogenized and homogenized milk reveal only slight differences in the concentration of these amino acids between the 2 membrane-protein preparations. Similar determinations for lysine, glutamic acid, tryptophan, cystine, and glycine show marked increases in the concentration of glutamic acid and lysine and decreases in cystine, glycine, and tryptophan in the membrane preparations from homogenized milk.

The amino acid composition of the membrane-protein preparations from nonhomogenized and homogenized milk differed markedly from any of the recognized milk proteins.

TABLE 1

A comparison of the essential amino acid, glutamic acid, cystine and glycine content of the fat-membrane protein fractions with milk and other recognized milk proteins (All values expressed as grams per 100 grams of anhydrous protein)

Constituent	Fat-membrane protein from			Minor-protein fraction[d]	Casein[d]	Lactalbumin[d]	β-lactoglobulin[d]	Pseudoglobulin[d]	Euglobulin[d]
	Nonhomogenized milk	Homogenized milk	Milk[c]						
Arginine	6.99	5.86	5.0	1.70	4.1	3.5	2.88	3.5	4.9
Cystine	1.50	0.68	1.5	0.86	0.34	4.1	2.29	3.0	3.2
Glycine	3.76	1.69	3.0	.....	0.5	.....	.....	.....	.....
Glutamic acid	12.85	17.80	.....	13.30	22.4	.....	19.50	.....	.....
Histidine	3.03	3.28	1.7	0.89	3.1	2.0	1.58	2.14	1.89
Isoleucine	5.67	6.00	3.5	3.66	6.1	.....	8.40	3.1	3.1
Leucine	8.71	9.22	9.0	5.71	9.2	15.0	15.60	9.1	10.4
Lysine	5.92	7.54	6.1	5.74	8.2	9.0	11.40	7.2	6.3
Methionine	2.08	2.20	2.0	1.34	2.8	2.8	3.22	1.08	0.98
Phenylalanine	4.97	4.51	5.2	2.35	5.0	5.6	3.50	3.8	3.6
Threonine	6.03	5.68	6.4	6.56	4.9	5.3	5.80	10.1	10.5
Tryptophan	1.72	1.14	0.9	0.87	1.2	2.3	1.94	2.7	2.4
Valine	5.65	5.50	5.4	9.74	7.2	4.0	5.80	9.4	10.4
Nitrogen (%)[e]	12.20	13.30	12.34	9.99	.....	.....	.....	.....	.....

[c] Experimental values reported by Hare, Schwartz, and Weese (*9*) for sample 1.

[d] Experimental and literature values reported by Weinstein, Duncan, and Trout (*36*).

[e] Actual nitrogen content of the anhydrous proteins.

An interesting comparison between the 2 membrane-protein preparations is attained through the calculation of their minimum molecular weights as based on the number of amino acid residues contained in the protein. The data used to make the (*M* min.) calculations are presented in Table 2. The calculated (*M* min.) values of 47,332 and 72,040 for the membrane-proteins of nonhomogenized and homogenized milk, respectively, represent average values obtained for 13 amino acids.

DISCUSSION

Isolation of the fat-membrane proteins. The question arises relative to the purity of the isolated protein preparations. The nitrogen values for washed cream reported in Figure 2 show that subsequent to the third washing practically all of the plasma protein has been diluted out of the system, because additional washings failed to lower the nitrogen/fat ratio. These data are not only in general agreement with those reported by Jack and Dahle *(11)*, but also demonstrate the existence of a significant difference in the fat/protein relationship between nonhomogenized and homogenized milk. The proteins which were recovered from the washed cream after 6 washings were considered to consist only of protein material closely associated with the fat globule surface.

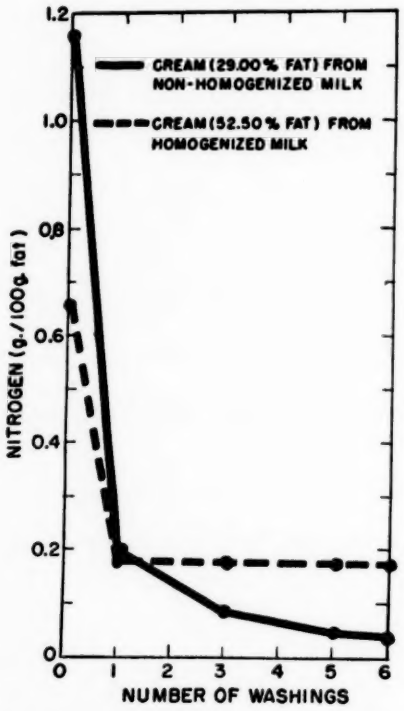

Figure 2. The effect of successive washings on the total nitrogent content of cream from nonhomogenized and homogenized milk.

No pretense is made, however, to infer that the proteins remaining on the fat globule, following 6 washings with warm water (50° to 55°C.), are representative, either qualitatively or quantitatively, of the proteins originally absorbed on, or associated with, the fat globule surface in nonhomogenized and homogenized milk. Palmer *(21)* has pointed out that the agitation and dilution effect involved in the washing of cream probably washes off all but the most tenaciously absorbed materials from the fat surfaces. Furthermore, the effect of the ethanol-ether treatment, necessary for the separation and removal of lipid materials, on the nature of the isolated proteins is not clearly understood at the present time. Nevertheless, the scheme for isolating fat-membrane proteins, as outlined herein, was the most satisfactory method obtained for preparing relatively lipid-free, fat-membrane proteins of high quality of the several methods employed.

Amino acid composition of fat-membrane proteins. Our present knowledge of the fat-membrane of nonhomogenized milk does not indicate clearly whether it is composed of a single protein or a mixture of proteins. Provided there was but a single protein component, then, the compositional data presented in Table 1 furnish significant information concerning the characteristics of the membrane-protein. If, on the other hand, the membrane is composed of a mixture of proteins, as may be the situation in the fat-membrane of homogenized milk, compositional data serve only a limited value. Their primary usefulness is to furnish data which indicate that the protein preparations isolated from nonhomogenized and homogenized milk differ in over-all composition. Significant differences would indicate that the normal membrane equilibrium had been altered by the homogenization process. In this respect the amino acid data show significant differences in the concentration of cystine, glycine, glutamic acid, and tryptophan between the membrane-proteins of nonhomogenized and homogenized milk; therefore, the assumption has been made that one or more of the protein components of the milk plasma had been absorbed on the newly created fat surfaces of homogenized milk.

With regard to the limitations already mentioned the calculated molecular weights presented in Table 2 become valid only upon the condition that the analyses were made on single proteins. If the fat-membrane protein of the nonhomogenized milk is a specific protein, then the calculated molecular weight for this material is of some value. Conversely, the calculated value for the membrane-proteins of homogenized milk is of little value since the probability is that the fat membrane is composed of a mixture of plasma proteins, absorbed during the homogenization process.

A certain degree of reliability has been added to the amino acid values presented in Table 1 by the recent report of Hare *et al. (9)* who, using the method of Olsen *(20)* for the isolation of phospholipid materials in milk, reported the concentration of 16 amino acids in the fat-globule membrane protein. Their data were obtained also by standard microbiological procedures and are partially tabulated in Table 1 for comparative purposes. With the exception of histidine, isoleucine, and tryptophan, the concentration of the other amino acids in the fat-membrane protein(s) isolated by them and in the membrane protein of nonhomogenized milk reported

TABLE 2

The number of amino acid residues in the fat-membrane proteins prepared by cold ethanol-ether extraction

Constituent	Fat-membrane proteins obtained from					
	Nonhomogenized milk			Homogenized milk		
	Yield	Moles per 10^5 g. protein	Molar ratio when tryptophan =	Yield	Moles per 10^5 g. protein	Molar ratio when tryptophan =
	(%)			(%)		
Arginine	6.99	40.1	19	5.86	33.7	24
Cystine	1.50	12.5	6	0.68	5.7	4
Glycine	3.76	50.1	24	1.69	22.5	16
Glutamic acid	12.85	87.4	41	17.80	121.0	90
Histidine	3.03	19.5	9	3.28	21.2	15
Isoleucine	5.67	43.3	20	6.00	45.8	33
Leucine	8.71	66.4	31	9.25	70.3	50
Lysine	5.92	40.5	19	7.54	51.6	37
Methionine	2.08	14.0	7	2.20	14.8	11
Phenylalanine	4.97	30.1	14	4.51	27.3	20
Threonine	6.03	50.6	24	5.68	47.7	34
Tryptophan	1.72	8.4	4	1.14	5.6	4
Valine	5.65	48.3	23	5.50	47.0	34
(*M.* min.)	.....	.....	47,332	.....	.....	72,010

in this study are of the same order. The existing discrepancies could conceivably arise from numerous sources, but probably constitute errors in methodology. Nitrogen values are of the same order and suggest that the fat-membrane protein preparations were similar to the materials isolated by Hattori *(10)* and Palmer *et al. (23, 38)*. The compositional agreement between the 2 samples of fat-membrane protein prepared by different methods and from milk produced in different sections of the country strengthens the proposition that the fat-membrane protein of normal milk is highly specific.

SUMMARY AND CONCLUSIONS

A procedure for isolating fat-membrane proteins from nonhomogenized and homogenized milk has been presented.

The microbiological assay for the essential amino acids, glutamic acid, cystine, and glycine in the fat-membrane protein of nonhomogenized and homogenized milk demonstrated material increases in the concentration of lysine and glutamic acid and marked decreases in the concentration of tryptophan, cystine, and glycine in the fat-membrane protein of homogenized milk. These differences in the amino acid composition indicate that the character of the fat-membrane protein of milk is changed as a result of homogenization and, further, that the main protein constituents of the milk plasma form a large percentage of the membrane-protein complex in homogenized milk.

Acknowledgment

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THE FAT-GLOBULE MEMBRANE OF NONHOMOGENIZED AND HOMOGENIZED MILK.
II. DIFFERENCES IN THE ELECTROPHORETIC PATTERNS OF THE FAT-MEMBRANE PROTEINS*

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Several investigations have been made to study the cataphoretic characteristics of fat globules whose electrokinetic mobility is determined largely by the nature of the material closely associated with the fat globule. Mohr and Brockmann *(8)* found the isoelectric point of fat globules in an acetate buffer to be about pH 4.3. They were not able to detect any change in the isoelectric point as a result of pasteurization or homogenization. North and Sommer *(11)*, using streaming potential measurements, determined the isoelectric point of fat globules to be about pH 4.3. Moyer *(9)* reported the isoelectric point of fat globules isolated from washed cream and normal milk to be pH 3.8 and 4.6, respectively. This observation indicated that some of the membrane material had been lost in the washing process. Dunkley and Sommer *(4)* reported an isoelectric point range of pH 4.3 to 4.5 for the normal fat globules of milk.

Jack and Dahle *(5, 6)* and Dahle and Jack *(3)* determined the isoelectric point of normal fat globules in a 1:200 dilution to be pH 4.3. Their data indicated a slight increase in the electrokinetic mobility for homogenized milk fat, *i.e.*, 2.53 u/sec./v./cm. for normal fat and 2.65 for homogenized fat. They found that the isoelectric point of milk fat that had been re-emulsified in a phospholipid sol to be about pH 2.0—the isoelectric point of phospholipid—whereas the addition of a casein sol, lactose, and salts shifted the isoelectric point toward that obtained for normal fat globules. From these data, they postulated the presence of a double-layer membrane on the surface of the fat globule, consisting of phospholipid and protein. Their studies represent the major portion of the experimental evidence which is available to show the effect of homogenization of the electrokinetic mobility of fat globules.

There are no data available, however, showing the electrokinetic characteristics of isolated membrane-proteins and only comparatively few electrophoretic studies have been made to characterize specifically the normal protein complement of milk.

In previous papers *(1, 2)*, the similarities and differences in the amino acid composition of the fat-membrane proteins isolated from non-homogen-

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ized and homogenized milk were reported. The purpose of this paper is to report the apparent difference between the Schlieren patterns of the fat-membrane proteins isolated from homogenized and nonhomogenized milk.

EXPERIMENTAL PROCEDURE

The fat-globule membrane proteins used in this study were a part of the same protein preparations reported previously *(2)*.

The electrophoretic characteristics of the lyophilized fat-membrane proteins were studied with the aid of the Perkin-Elmer, Model 38, Tiselius electrophoresis apparatus described by Moore and White *(10)*. The principle of its operation is based on Longsworth's scanning modification of the Toepler-Schlieren method. A Tiselius-type cell with a capacity of 2 ml. and a cross-sectional area of 0.3 sq. cm. was used to hold the protein solution in which the moving boundaries of the various protein components were formed.

Solutions of the fat-membrane proteins were prepared for electrophoresis by adding exactly 0.1 g. of the lyophilized sample to 10 ml. of the desired buffer in a stoppered test tube. The contents of the tube were mixed and allowed to stand for approximately 24 hr. at 18°C. The protein-buffer solution was transferred to a cellophane sac and dialyzed with continuous agitation against 600 ml. of the buffer, maintained at 5°C. for 6 hr., to obtain constant conductivity on both sides of the cellophane membrane. The protein buffer solution was then centrifuged at 2,000 r.p.m. for approximately 1 hr. A portion of the supernatant was used for electrophoresis at 1.2°C. The concentration of the fat-membrane protein in the equilibrated solution was based on nitrogen values of 12.20 and 12.30% for nonhomogenized and homogenized milk membrane-proteins, respectively. Nitrogen was determined by a standard micro-Kjeldahl technique.

The membrane-protein samples were characterized electrophoretically in the following buffer systems: glycine-HCl, pH 1.5; phosphate, pH 6.5; veronal-citrate, pH 8.0; ammonia-HCl, pH 9.0; and veronal-ethylamine, pH 10.8. The buffers were calculated to be approximately 0.1 ionic strength.

The slight opacity of the homogenized milk membrane-protein solutions made it necessary to use Kodak Super Panchro-Press, type B, film to record the electrophoretic patterns. All other patterns were recorded with Kodak Contrast Process Panchromatic film.[e] The photographic plates were retouched to remove evidence of opalescence and light streaking and improve the clarity of the mobility patterns. Precautions were taken, however, not to distort the dimensions of the original patterns.

RESULTS

Electrophoretic mobility patterns for the cold ethanol-ether extracted fat-membrane proteins in the selected pH and buffer media are illustrated in Figure 1. Both the ascending and descending mobility patterns are shown. The calculated mobilities and relative areas of the migrating boundaries which are shown in the descending-channel patterns in Figure 1 are tabulated in Table 1. Because of the preliminary nature of this study and the uncertainty of the location or existence of a so-called "false boundary," the data presented in Table 1 have not been corrected according to the recommendations of Longsworth, Curtis, and Pembroke *(7)*. In lieu of these corrections the descending-channel measurements offer the better values for making mobility calculations. The peak designators used in Table 1 are similar to those used in Figure 1 and do not represent, necessarily, the same component in all of the electrophoretic patterns.

Where good electrophoretic resolution of the protein components occurred, 2 and sometimes 3 prominent and distinct peaks were observed in the electrophoretic patterns of the fat-membrane proteins prepared from nonhomogenized milk. These data are representative of the normal fat-membrane proteins. Similarly, the patterns from the homogenized fat-membrane proteins showed 3 or 4 distinct peaks. In most instances, however, there appeared to be a distinct difference in the basic characteristics of the electrophoretic patterns of the fat-membrane proteins prepared from nonhomogenized and homogenized milk.

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[e] Mention of products of commerce in Food Research does not imply indorsement over other products of the same nature.

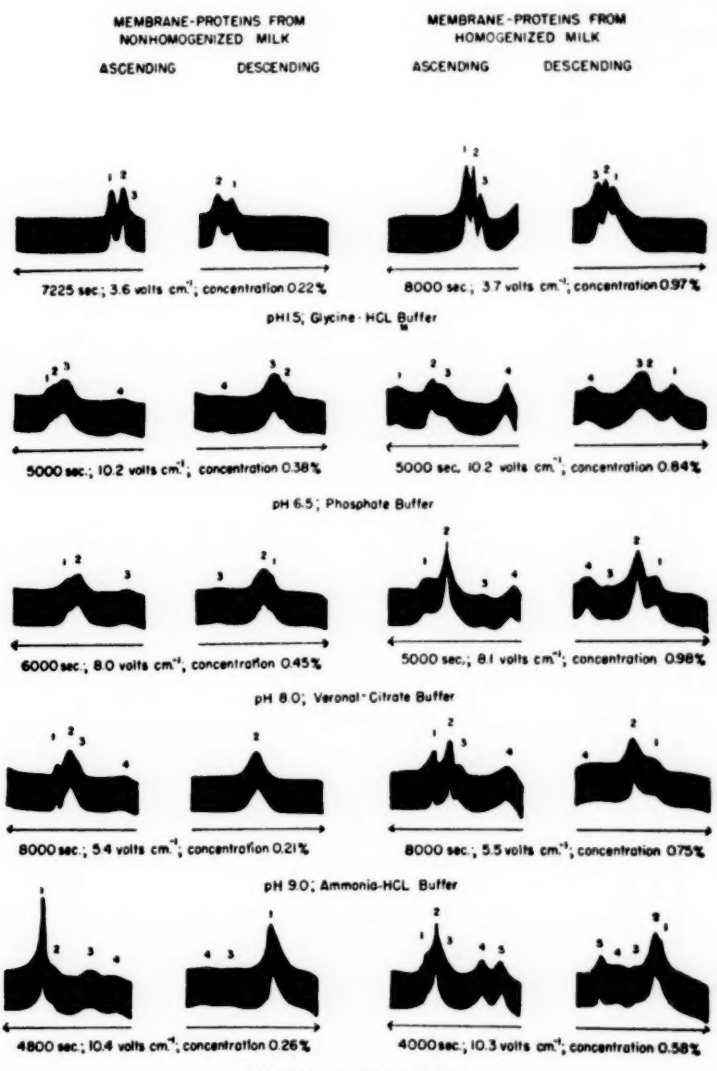
MEMBRANE-PROTEINS FROM NONHOMOGENIZED MILK
ASCENDING
DESCENDING
MEMBRANE-PROTEINS FROM HOMOGENIZED MILK
ASCENDING
DESCENDING
7225 sec.; 3.6 volts cm.⁻¹; concentration 0.22%
8000 sec.; 3.7 volts cm.⁻¹; concentration 0.97%
pH 1.5; Glycine-HCL Buffer
5000 sec.; 10.2 volts cm.⁻¹; concentration 0.38%
5000 sec. 10.2 volts cm.⁻¹; concentration 0.84%
pH 6.5; Phosphate Buffer
6000 sec.; 8.0 volts cm.⁻¹; concentration 0.45%
5000 sec.; 8.1 volts cm.⁻¹; concentration 0.98%
pH 8.0; Veronal-Citrate Buffer
8000 sec.; 5.4 volts cm.⁻¹; concentration 0.21%
8000 sec.; 5.5 volts cm.⁻¹; concentration 0.75%
pH 9.0; Ammonia-HCL Buffer
4800 sec.; 10.4 volts cm.⁻¹; concentration 0.26%
4000 sec.; 10.3 volts cm.⁻¹; concentration 0.58%
pH 10.8; Veronal-Ethylamine Buffer

Figure 1. Electrophoretic mobility patterns of the fat-membrane protein(s) of nonhomogenized and homogenized milk in various buffers and pH levels.

DISCUSSION

The individual characteristics of the mobility patterns of the two protein preparations vary sufficiently to assume that their constituent protein components likewise vary. Conceivably, the protein components appearing

TABLE 1

Migration distances, mobilities, and relative percentages area of fat-membrane protein components in various buffers as determined from the descending-channel electrophoretic mobility patterns shown in Figure 1. [Mobility (u) = cm. , sec. , V. , × 10]

Peak No.[a]	Buffer system ($\Gamma/ = 0.1$)														
	Glycine-HCl pH 1.5			Phosphate pH 6.5			Veronal-citrate pH 8.0			Ammonia HCl pH 9.0			Veronal-ethylamine pH 10.8		
	d cm.[b]	*u* (+)	A%	*d* cm.	*u* (−)	A%	*d* cm.	*u* (−)	A%	*d* cm.	*u* (−)	A%	*d* cm.	*u* (−)	A%
Nonhomogenized milk															
1	1.05	4.04	41	....	....	....	2.52	5.27	31	....	....	....	2.87	5.74	93
2	0.56	2.16	59	3.12	6.12	30	2.30	4.81	66	2.22	5.11	100	....	....	....
3	....	....	....	2.78	5.45	69	0.76[f]	1.59	3	....	....	....	1.50	3.00	2
4	....	....	....	0.90[f]	1.76	2	....	....	....	....	....	....	0.51[f]	1.02	5
Homogenized milk															
1	1.31	4.40	46	3.41	6.68	21	2.83	6.99	22	2.53	5.77	26	2.82	6.82	41
2	1.04	3.49	23	2.33	4.57	25	2.12	5.24	63	....	....	....	2.64	6.39	39
3	0.79	2.65	31	2.12	4.16	42	1.13	2.79	1	1.87	4.26	71	1.90	5.00	1
4	....	....	....	0.44[f]	0.86	12	0.45[f]	1.11	14	0.73[f]	0.84	3	1.38	3.34	6
5	....	....	....	....	....	....	....	....	....	....	....	....	0.84[f]	2.03	13

[a] Designation of peaks shown in Figure 1.
[b] Measured from initial boundary.
[f] Possible false boundaries.

in the mobility patterns of the membrane proteins from nonhomogenized milk could have been masked or their relative prominence occluded in the mobility patterns of the homogenized membrane proteins. Such a condition could arise easily as a result of the diluting effect of newly adsorbed protein components on the newly created surface of homogenized milk fat. Thus, the 3 or 4 protein components observed in the mobility patterns of homogenized fat-membrane proteins could be adsorbed proteins which were not representative of any of the protein components observed in the mobility patterns of the nonhomogenized fat-membrane proteins.

The authors have not used the data presented herein in an attempt to aid in the identification of protein systems studied. This possibility has been avoided, primarily, because of the preliminary nature of the reported data and of the unique character of the protein systems under study. They do not represent the normal milk protein complement as one finds in milk, skimmilk, whey or solution of purified proteins. Consequently, the authors believe that a direct comparison with the existing electrophoretic data at this time might prejudice a subsequent attempt to identify the protein components at a time when the experimental conditions are more favorable. However, it is conceivable to expect that all of the milk plasma proteins are capable of being adsorbed on the fat-globule surface and probably were adsorbed on the newly created fat surfaces in homogenized milk.

The opacity observed in the dialyzed membrane-protein solutions of homogenized milk, on several occasions, resulted in partially under-exposed "Schlieren" electrophoretic patterns. This defect has been observed by Longsworth *et al. (7)* in blood serum and was attributed to the incomplete extraction of lipid material from the serum. Tobias, Whitney, and Tracy *(14)* showed that skimmilk with a fat content of 0.1% absorbed so much light that electrophoretic "Schlieren" photography became quite difficult. In the experiments reported here the maximum light absorption preceded the main component on the descending patterns and followed the main component on the ascending patterns. This observation may or may not indicate that the lipid material present in the protein-buffer solution was closely associated with the main protein component. Tobias *et al. (14)* fixed the boundary between the over-exposed and under-exposed portion of the photographic film at the β-lactoglobulin boundary in the electrophoretic mobility patterns of skimmilk at pH 6.8 and 8.7.

In many of the electrophoretic "Schlieren" patterns shown in Figure 1 the major protein components appear to be complexes or closely associated proteins of quite similar electrophoretic characteristics. The possibility for the existence of milk protein complexes, both naturally occurring and those produced as a result of processing treatments, has been suggested by Stanley, Whitnah, and Andrews *(13)*, Tobias, Whitney, and Tracy *(15)*, and Slatter and Van Winkle *(12)*.

SUMMARY AND CONCLUSION

Ascending and descending electrophoretic mobility patterns are reported for the fat-membrane proteins prepared from nonhomogenized and homogenized milk in the following buffer systems: glycine-HCl, pH 1.5; phosphate, pH 6.5; veronal-citrate, pH 8.0; ammonia-HCl, pH 9.0; veronal-

ethyl-amine, pH 10.8. Values for the electrophoretic mobilities and relative areas of the component peaks in the descending-channel patterns were calculated.

Generally, one to 3 components were observed in the nonhomogenized fat-membrane proteins, whereas 2 to, possibly, 4 components of different electrophoretic characteristics were observed in the homogenized fat-membrane proteins.

No positive identification of these components was attempted from the existing electrophoretic evidence, but quite conceivably, the protein complement of the fat-membrane of homogenized milk is composed of adsorbed milk plasma proteins in addition to its normal complement of the fat-membrane protein complex.

Acknowledgment

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THE FAT-GLOBULE MEMBRANE OF NONHOMOGENIZED AND HOMOGENIZED MILK.

III. DIFFERENCES IN THE SEDIMENTATION DIAGRAMS OF THE FAT-MEMBRANE PROTEINS*

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Samples of the fat-membrane proteins of nonhomogenized and homogenized milk have been isolated and differentiated on the basis of their amino acid composition *(2)* and on the basis of their electrophoretic properties *(3)*. Further characterization and resolution of the fat-membrane proteins by means of sedimentation in an ultracentrifuge is desirable.

A limited amount of very carefully compiled sedimentation data are available on several of the protein components of normal milk. Svedberg, Carpenter, and Carpenter *(12, 13)*, using the sedimentation equilibrium method, reported the molecular weight and other physical aspects of the Hammersten and the Van Slyke and Baker casein. Pedersen *(8)* was able to demonstrate by ultracentrifugal data that casein-free milk serum contained three distinct protein components which he designated as α, β, and γ protein. The α-component is a substance of low molecular weight isolated by Kekwick, the β-component is the lactoglobulin of Palmer, and the γ-component is the protein usually designated as lactoglobulin. Smith *(10)*, by similar techniques, observed 2 to 3 components in bovine whey, one of which he believed to be lactoglobulin.

Further ultracentrifugal studies by Pedersen *(9)* and others *(4, 6)* showed that Palmer's β-lactoglobulin was homogeneous with a molecular weight of about 39,000. Ogston *(7)* reported sedimentation data for Aschaffenburg's *(1)* sigma-proteose, a protein-like component of the albumin- and globulin-free whey. Deutsch *(5)* reported sedimentation diagrams of whey proteins from the milk of several mammals at various times post partum. These reports, which represent a portion of the sedimentation data available on the protein components of milk, illustrate the usefulness of ultracentrifugal data for the purpose of establishing partially the chemical and physical identity of the protein complement of milk.

This paper is concerned with the differentiation of the fat-membrane proteins obtained from nonhomogenized and homogenized milk by applying the ultracentrifugal sedimentation-velocity method to the respective membrane protein solutions.

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EXPERIMENTAL PROCEDURE

An electrically driven Model E, Spinco ultracentrifuge, described in detail by Smith, Brown, Fishman, and Wilhelmi *(11)*, was used to obtain the sedimentation diagrams of the fat-membrane proteins of nonhomogenized and homogenized milk. The membrane proteins used in this study were procured from the same lots prepared and used in previous work *(2, 3)*.

Solutions of the fat-membrane protein preparations were made to approximately 2% in veronal-citrate buffer at a pH of 8.0 and an ionic strength of 0.09. Twenty liters of buffer were made by dissolving 45 g. of sodium barbital in 2 l. of distilled water which was adjusted to pH 8.0 with citric acid. This base solution was diluted to 20 l. of buffer solution.

Although maximum dispersion of the fat-membrane proteins was attained in this buffer system, there was a considerable degree of opacity in the solutions. After standing for 24 hr. at approximately 10°C., the solutions were centrifuged at room temperature for 1 hr. at 2,500 r.p.m. in a clinical-type centrifuge to remove the suspended protein materials. A portion of each supernatant was saved for the ultracentrifugal analysis and a subsequent nitrogen determination. Approximately 0.8 ml. of the protein solution was required to fill the analytical cell to the prescribed operating level. A rotor speed of 47,660 r.p.m. was arbitrarily chosen for this study. Upon reaching the desired speed, the angle of the inclined bar was adjusted to 50°. Sedimentation diagrams were recorded at selected time intervals on Kodak Spectroscopic photographic plates (Type 1-D) with the use of a Wratten No. 16 (yellow) filter. Sedimentation constants of the recorded diagram peaks were calculated according to the methods of Svedberg and Pedersen *(14)* and were expressed as Svedberg units ($S_{20°C.} = s_{20} \times 10^{-13}$); uncorrected for solvent effect.

The percentages of protein in the centrifuged samples were based on nitrogen values of 12.20 and 13.30% for nonhomogenized and homogenized milk membrane-proteins, respectively. Nitrogen was determined by a standard micro-Kjeldahl technique. Nonprotein nitrogen was considered negligible.

RESULTS

Sedimentation diagrams for the nonhomogenized (1.41%) and homogenized membrane-protein (1.83%) solutions are shown in Figures 1 and 2, respectively. The sedimentation diagrams of the fat-membrane protein from nonhomogenized milk showed one principal component of quite homogeneous nature, $S_{20} = 7.3$. There was some indication of a small amount of slightly heavier material which became quite diffuse after 49 min. of centrifugation with no further resolution occurring. Similar sedimentation diagrams for the fat-membrane protein of homogenized milk showed that there were 3 distinct, and possibly a fourth protein component present. Two of the components, peak 2, $S_{20} = 8.2$, and peak 3, $S_{20} = 4.4$, which were present in low concentration moved to the bottom of the analytical cell fairly rapidly. The main component, peak 1. $S_{20} = 2.2$, moved through the cell rather slowly and was followed by a much slower moving component, peak 4, which was manifested as a very diffuse pattern in the sedimentation diagram.

DISCUSSION

The sedimentation diagrams of the fat-membrane proteins of nonhomogenized and homogenized milk show distinct differences in protein complement, as do their electrophoretic patterns *(3)*. Since sedimentation and electrophoretic characteristics are dependent upon different properties of the protein, the data presented herein may further support the conjecture that the homogenized fat globule has been induced to adsorb proteins other than those occurring normally on the fat-globule membrane.

The primary boundary in the sedimentation diagram of nonhomogenized milk membrane-protein was characterized by a sedimentation velocity of 7.3 Svedbergs. The sedimentation velocity of the predominant boundary in the homogenized milk membrane-protein was 2.2 Svedbergs. The remain-

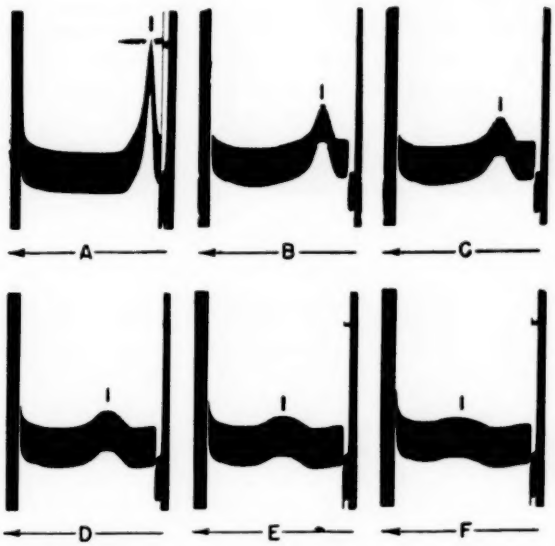

Figure 1. Sedimentation-velocity diagrams for the fat-membrane proteins obtained from nonhomogenized milk. Concentration, 1.41% in veronal-citrate buffer at pH 8.0 and ionic strength of 0.09; angle of bar to the slit image, 50°; average temperature, 23.7°C.; speed, 47,660 r.p.m.; time interval after exposure A, B = 20 min., C = 32 min., D = 49 min., E = 65 min., and F = 81 min.

ing boundaries in the sedimentation diagram of the homogenized milk sample had sedimentation values of 8.2 and 4.4 Svedbergs. These observations indicate that the major protein constituent of the fat membrane of nonhomogenized milk was not recovered in the protein preparation from its homogenized counterpart. However, the apparent absence of the normal fat-membrane protein may quite conceivably be explained on the basis of a dilution effect, since the fat surface is increased approximately 5-fold by homogenization, or on the basis of physical and/or chemical alteration of the protein constituents as a result of homogenization. Differences arising as a result of alterations in the physical-chemical properties due to pasteurization must be excluded since both membrane-preparations were isolated from milk obtained from the same lot of pasteurized milk. Nevertheless, the pasteurization process may partially obscure the identity of the protein components appearing in the sedimentation diagrams, thus making uncertain a direct comparison with the sedimentation values of recognized milk proteins.

Sedimentation values reported in the literature for various milk proteins offer some clue to the nature of the adsorbed fat-membrane protein. Sedimentation values approximating 7 Svedbergs have been reported for the classical lactoglobulin *(5, 8, 10)*. Sedimentation constants ranging from 3 to 23 Svedbergs, depending upon the conditions of the experiment and the treatment of the milk sample, have been reported for the casein frac-

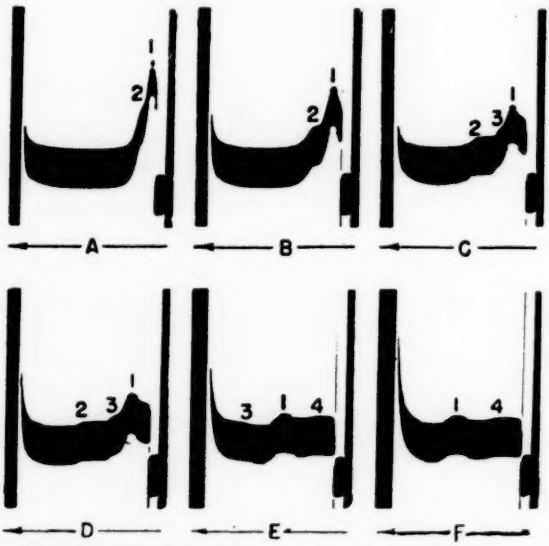

Figure 2. Sedimentation-velocity diagrams for the fat-membrane proteins obtained from homogenized milk. Concentration, 1.83% in veronal-citrate buffer at pH 8.0 and ionic strength of 0.09; angle of bar to the slit image, 50°; average temperature, 22.6°C.; speed, 47,660 r.p.m.; time interval after exposure A, B = 16 min., C = 40 min., D = 57 min., E = 174 min., and F = 222 min.

tion of the milk proteins *(8, 12, 13)*. The β-lactoglobulin of Palmer has been characterized ultracentrifugally and has been assigned sedimentation values ranging between 2.8 and 3.2 Svedbergs *(5, 8, 9)*. Kekwick isolated a protein component from whey which was characterized by sedimentation constants ranging between 1 and 2 Svedbergs *(5, 8)*. In addition to these major protein constituents of milk, Ogston *(7)* has reported sedimentation values of 2.8 and 1.0 for 2 components of a minor protein complex which was isolated from whey by Aschaffenburg *(1)*. Because of the relationship of the determined sedimentation values to experimental conditions, a direct comparison of the sedimentation values reported in this study with the sedimentation values for the protein constituents reported in the literature must be provisional.

By making a liberal interpretation of the existing sedimentation data the normal fat-membrane protein (Fig. 1) might be classified as lactoglobulin. The sedimentation value of component No. 1 ($S_{20} = 2.2$), apparent in the sedimentation diagram for the homogenized milk membrane-protein (Fig. 2), agrees very favorably with the sedimentation value of the whey protein isolated by Kekwick *(8)*. In the same material, component No. 2 ($S_{20} = 8.0$) could be a casein or a fraction of the casein complex. The possibility exists that this component may represent the milk lactoglobulin which has been altered by the effect of homogenization. The sedimentation value for component No. 3 ($S_{20} = 4.4$) does not compare closely with any

of the reported sedimentation values for the classical milk proteins. Conceivably, this boundary might represent Palmer's β-lactoglobulin, altered as a result of homogenization and/or adsorption at the fat surface. Then, there is the possibility that 2 of the slower moving components, namely, Kekwick's whey protein and/or β-lactoglobulin, may have complexed with the faster moving lactoglobulin or casein. The slow moving component (No. 4, $S_{20} =$ diffuse), which is vaguely apparent in the sedimentation diagrams of the homogenized milk membrane-protein, could be the minor protein reported by Aschaffenburg *(1)*. The occurrence of this component on the resurfaced fat in homogenized milk would be expected on the basis of its reported high surface activity.

SUMMARY AND CONCLUSIONS

Ultracentrifugal sedimentation-velocity diagrams for the fat-membrane proteins of nonhomogenized and homogenized milk in veronal-citrate buffer at pH 8.0 and an ionic strength of 0.09 are reported. Sedimentation values for the protein components are expressed in Svedberg's ($S_{20°C.} = s_{20} \times 10^{-13}$).

The sedimentation diagrams show one principal and fairly homogenous protein component ($S_{20} = 7.3$) in the fat-membrane protein preparation of nonhomogenized milk, whereas three ($S_{20} = 2.2$, 8.2, and 4.4) components and possibly one of lower concentration and mobility were detected in the fat-membrane proteins of homogenized milk.

The protein complement of the fat-globule membrane of milk is altered by homogenization.

The fat-membrane protein was classified provisionally as lactoglobulin or a protein of globulin-like nature.

The proteins which constitute the adsorbed proteins in homogenized milk were identified provisionally as casein, Kekwick's whey-protein, and an altered lactoglobulin, β-lactoglobulin or a complex of 2 or more of the constituent proteins.

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PARTICIPATION OF ASCORBIC ACID IN THE DESTRUCTION OF ANTHOCYANIN IN STRAWBERRY JUICE AND MODEL SYSTEMS*

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That ascorbic acid may play a significant role in the red color deterioration of fruit juices was first pointed out by Beattie, Wheeler, and Pederson *(2)* and by Pederson, Beattie, and Stotz *(6)* who observed parallel losses of ascorbic acid and anthocyanins during storage. The best correlation between ascorbic acid and red color losses was obtained with strawberry juice. Pederson and coworkers postulated that an interaction between ascorbic acid and anthocyanins may occur. Similar observations and conclusions were reported by Nebesky, Esselen, McConnel, and Fellers *(5)*.

The availability of crystalline pelargonidin 3-monoglucoside isolated from strawberries *(10)* prompted us to study these interactions in simplified artificial systems as well as in strawberry juice. In the presence of ascorbic acid, maximum anthocyanin destruction was observed under those conditions which favor the aerobic oxidation of ascorbic acid.

EXPERIMENTAL

Pelargonidin 3-monoglucoside chloride was obtained by a previously described procedure *(10)*. The anthocyanin picrate was recrystallized six times from hot dilute picric acid solution before conversion to the chloride. The model systems contained 28 mg. pigment per 100 ml. of Sorensen's disodium citrate-hydrochloric acid buffer of pH 3.40. The strawberry juice used in these experiments was expressed from Dresden strawberries grown at Geneva, N. Y., during the 1950 season. This juice had a pH of 3.4 and an ascorbic acid and anthocyanin content of 49 mg. and 23 mg. per 100 ml., respectively. All samples were heated to 90°C., and held for 5 minutes in order to expel most of the dissolved gases. The solutions which were to be held under essentially anaerobic conditions were rapidly poured into 2-dram (7.39 ml.) screw-capped vials, leaving no headspace. They were cooled in a 30°C. constant-temperature bath and sealed with paraffin wax. In experiments where a fairly constant air supply was desired, 50 ml. of solution in a 250-ml. volumetric flask was shaken continuously in an automatic shaker in which the temperature could be controlled.

Anthocyanin losses were followed by a previously described method *(9)* based on the difference in absorption at 500 mμ produced by a change in pH from 3.4 to 2.0. Ascorbic acid was determined by the method of Robinson and Stotz *(7)* which involves oxidation by indophenol under specific conditions. The above authors warn against the possible interference of sulfhydryl compounds in the ascorbic acid determination. We found that thiourea does tend to give high ascorbic acid values but that the error is small if the thiourea content does not exceed 0.1% and the excess indicator is extracted rapidly.

RESULTS AND DISCUSSION

Data on the rate of anthocyanin destruction in aerobic model systems are presented in Figure 1. Pigment destruction in solutions containing

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no ascorbic acid was very slow, curve A, Figure 1. However, on the addition of ascorbic acid a rapid color loss ensued which was accompanied by rapid ascorbic acid oxidation, curves B and B_1, respectively. That this color loss is related to the *rate of ascorbic acid oxidation* was demonstrated in two ways.

I. In anaerobic systems containing 50 mg. ascorbic acid per 100 ml., the anthocyanin loss was not significantly different from that observed in aerated solutions containing no ascorbic acid. After storage at 30° for 160 hours the pigment losses were 6% and 4%, respectively. Therefore, when lack of oxygen is inhibiting the ascorbic acid oxidation no demonstrable anthocyanin deterioration can be attributed to the ascorbic acid.

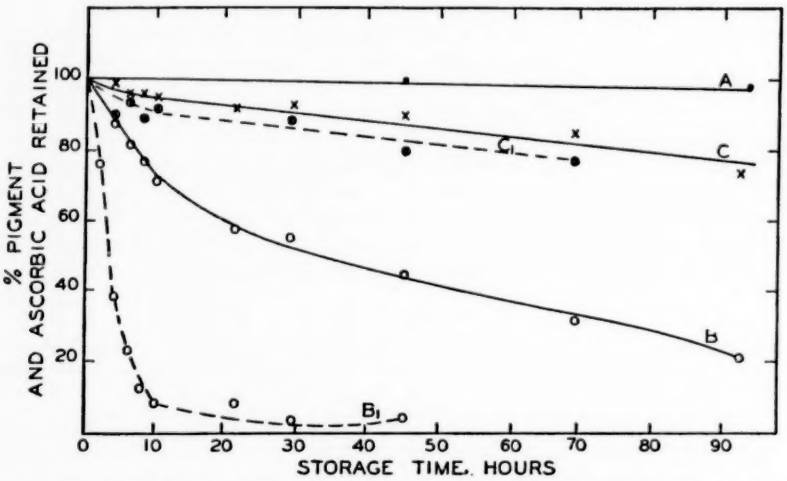

Figure 1. Pigment and ascorbic acid retention in model systems at 30°C. in the presence of air. Legend: A ·———· = pigment retention in systems containing no ascorbic acid. B ○———○ = pigment retention in systems containing 50 mg. ascorbic acid per 100 ml. C ×———× = pigment retention in systems containing 50 mg. ascorbic acid per 100 ml. and 100 mg. thiourea per 100 ml. B_1 ○- - - -○ = ascorbic acid retention in systems containing 50 mg. ascorbic acid per 100 ml. C_1 ⊗- - - -⊗ = ascorbic acid retention in systems containing 50 mg. ascorbic acid per 100 ml. and 100 mg. thiourea per 100 ml.

II. Thiourea is a metal-complexing agent which has previously been used to decrease the rate of cupric ion-catalyzed, aerobic oxidation of ascorbic acid *(4)*. Thiourea in the absence of ascorbic acid has no demonstrable effect on the rate of pigment destruction. Although no cupric ions were added to our systems, they may be assumed present in trace quantities as impurities. Barron, De Meio, and Klemperer *(1)* had to resort to extremely stringent purification techniques to remove the last traces of copper before oxygen-stable ascorbic acid solution could be prepared. From curves C and C_1 it may be seen that the addition of thiourea not only decreased the rate of ascorbic acid oxidation, but also decelerated the rate of

anthocyanin destruction. From this it is concluded that the rate of anthocyanin loss is influenced by the rate of ascorbic acid oxidation.

It has been demonstrated that hydrogen peroxide is formed during the cupric ion-catalyzed aerobic oxidation of ascorbic acid *(3, 8)* and that hydrogen peroxide, even in low concentrations, can oxidize pelargonidin 3-monoglucoside into less intensely colored compounds *(11)*. It appears likely therefore that hydrogen peroxide, or related substances, may be in part responsible for the loss of anthocyanin under the conditions of these experiments. That other factors are also involved is indicated by the continued, albeit slower, rate of loss of anthocyanin even after all the ascorbic acid has been oxidized.

The same relation between the rates of ascorbic acid oxidation and anthocyanin destruction in the presence of air could be demonstrated with strawberry juice as with model systems. These results are presented in Figure 2. The addition of 0.1% thiourea decreased the rate of ascorbic acid oxidation and slowed the rate of pigment decolorization.

Other workers have shown that headspace has an undesirable effect on the stability of the red pigments of strawberry juice. By comparing curve B, Figure 2 and curve B, Figure 3, it may be seen that our experiments confirm this observation. Our results indicate that color loss is not due to a direct oxidation of the pigment by air since pelargonidin 3-monoglucoside is fairly stable at a pH of 3.4 when shaken with air. However, when ascorbic acid is added to the above system, a rapid decolorization occurs. It

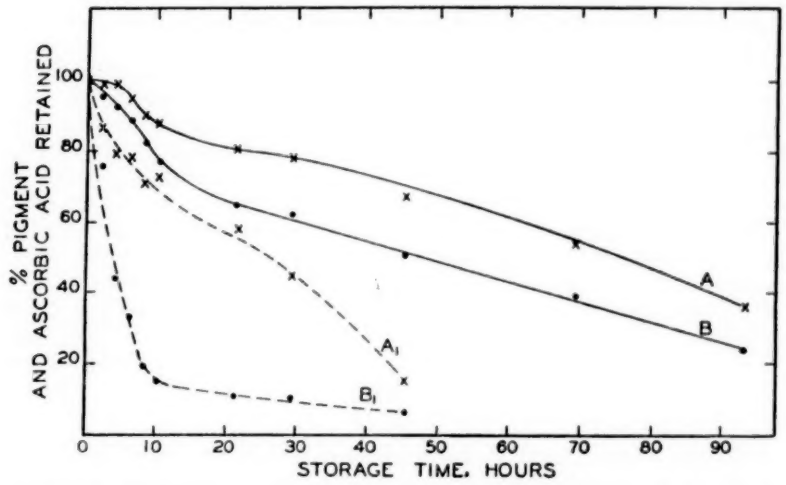

Figure 2. Pigment and ascorbic acid retention in strawberry juice at 30°C. in the presence of air. Legend: A ×———× = pigment retention in juice containing 49 mg. ascorbic acid per 100 ml. and 100 mg. per 100 ml. thiourea. B ·———· = pigment retention in juice containing 49 mg. ascorbic acid per 100 ml. without thiourea. A_1 ×----× = ascorbic acid retention in juice containing 49 mg. ascorbic acid per 100 ml. and 100 mg. thiourea per 100 ml. B_1 ·-----· = ascorbic acid retention in juice containing 49 mg. ascorbic acid per 100 ml. without thiourea.

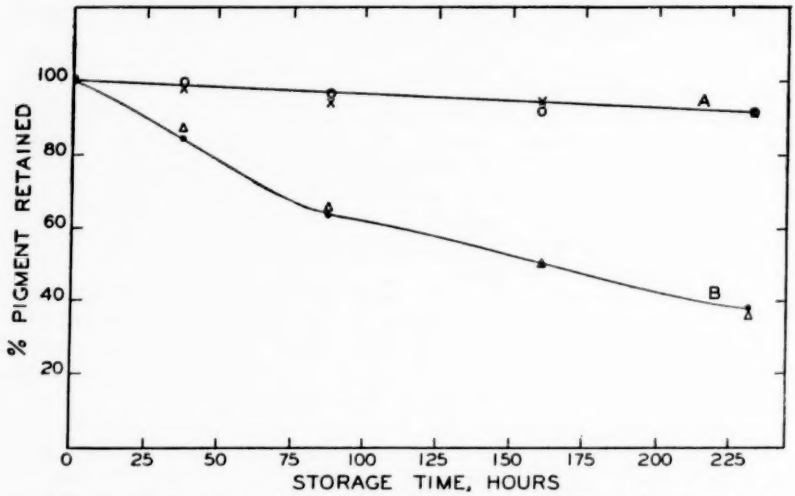

Figure 3. Pigment retention in model systems and strawberry juice at 30°C. in the absence of air. Legend: A = pigment retention in model systems, ○———○ no ascorbic acid, ×———× 50 mg. of ascorbic acid per 100 ml. B = pigment retention in strawberry juice, △———△ no added thiourea; ·———· 100 mg. thiourea per 100 ml.

is therefore likely that the harmful effect of headspace on color stability in strawberry juice may be attributed largely to the oxidation of ascorbic acid.

In experiments conducted with model systems and strawberry juice in the absence of air, the relation between ascorbic acid and anthocyanin losses is more difficult to evaluate. With model experiments no evidence for ascorbic acid-induced, anaerobic color loss was obtained, curve A, Figure 3. Similarly, in the absence of air, the rate of pigment destruction in strawberry juice to which thiourea had been added was not significantly different from that found in juice containing no added thiourea, curve B, Figure 3. It is noteworthy though that in strawberry juice the anthocyanin is destroyed at a much more rapid rate than in the model systems in spite of the fact that the ascorbic acid content and pH are identical in the 2 systems. This is shown in Figure 3. It seems probable that in the absence of air pigment destruction in strawberry juice proceeds primarily by yet unknown mechanisms not involving ascorbic acid.

SUMMARY

Evidence is presented for an indirect ascorbic acid-induced destruction of the anthocyanin pelargonidin 3-monoglucoside. Conditions which decrease the rate of the oxidation of ascorbic acid, lack of oxygen and the addition of thiourea, also decelerate the rate of anthocyanin destruction. Hydrogen peroxide, or related substances, are believed to play a significant role in this process.

The ascorbic acid-induced pigment destruction is believed to explain the harmful effect of headspace on pigment stability in strawberry products.

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OBSERVATIONS ON REDUCING GROUPS AND COLOR CHANGES IN MACARONI DISCS*

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While searching for an explanation of the effect of pressure in promoting translucency in macaroni discs, Fifield, Smith, and Hayes *(3)* determined the moisture content of pressed and unpressed discs. These workers noted that the pressed discs turned a golden brown while the unpressed discs were unchanged in color. Fifield *et al.* attributed this change to caramelization of dextrins formed from the starch by partial hydrolysis induced by pressure. Pressed discs suffered less moisture loss during heating.

Later work carried out in our own laboratory showed that there is a direct relation between pressure intensity and color development as well as between pressure and moisture content of macaroni discs. Figure 1 provides information on the effect of various pressures on moisture content as influenced by sodium chloride, acetic acid, and a wetting agent. Increase in pressure generally tended to reduce loss of moisture, with the greatest effect occurring below 3,000 lbs. Pressure thus appeared to alter the bound water relationship of the macaroni dough causing more water to be retained when pressure was applied. Sodium chloride was the most effective in retaining moisture at all pressures; the other agents employed exerted little influence. This difference probably lies in the ionic activity of the sodium chloride which affected the water retention of the disc.

The moisture content of the pressed material was related to browning. It was thought at first that the reaction might be enzymatic, probably amylolytic, but the addition of amylases to the macaroni dough was without noticeable effect on the color of the dried discs. It was then surmised that a typical browning reaction was taking place, caused by a protein-carbohydrate reaction. This paper is the result of a study to ascertain the basic causes of this phenomenon.

Numerous papers have been published on the formation of colored compounds by interaction between amino acids and carbohydrates. Hlynka *(7)* and Hlynka and Bass *(9)* postulated that reducing carbohydrates function as cross-linking agents between protein molecules. Increases in length of storage period, temperature, and moisture content favored the production of reducing groups. Hlynka and Anderson *(8)* and Hlynka and Bass *(9)* showed that a reaction occurs between proteins and carbohydrates in flour and dried dough after storage for 6 months.

Several explanations have been suggested to account for the reaction. Halton and Blair *(5)* and Sullivan *et al. (17)* thought that disulfide linkages are involved. In wheat, which contains cystine, 2 free thiol groups

*Contribution from the Department of Cereal Technology, North Dakota Agricultural College.

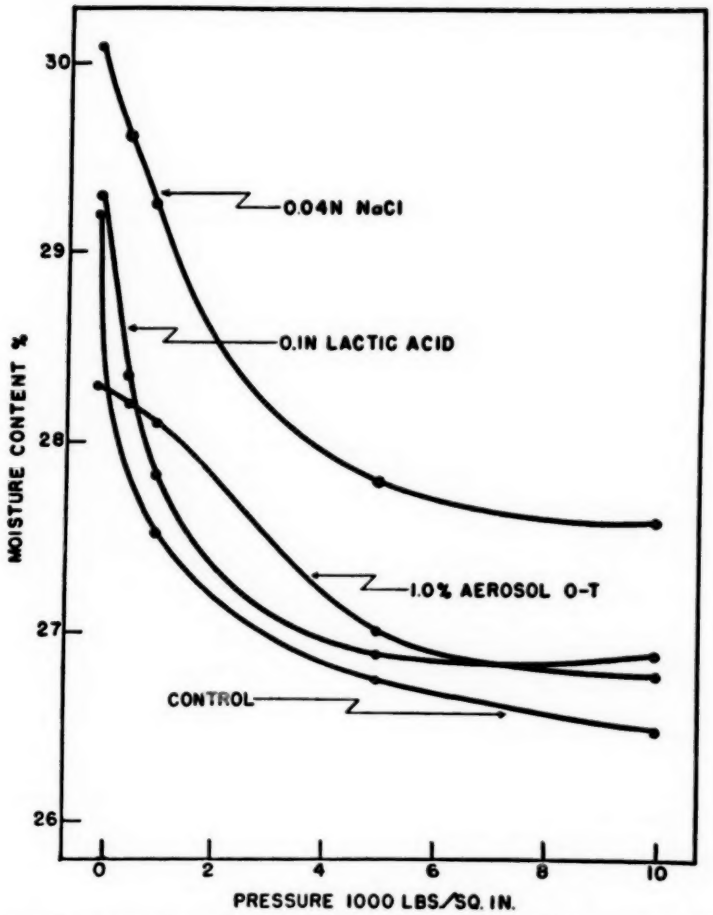

Figure 1. Effect of pressure and various substances on the moisture content of disc macaroni after drying for one hour at 130°C.

might react with glucose *(8, 9, 14)*. Schubert *(15)* showed that cysteine combined with arabinose, glucose, mannose, and lactose in a mole-to-mole ratio with the elimination of water.

An alternative method of combination is through a free amino group, as suggested by Mohammed, Fraenkel-Conrat, and Olcott *(11)* from a study of the browning reaction of bovine serum albumin in albumin-glucose solutions. Rate of loss of detectable free amino groups changed from 1.2% to 0.1% of amino nitrogen in 13 days.

Hlynka *(7)* found that acetaldehyde prevents dough deterioration by sodium bisulfite when present in a mole ratio of 1 to 24. It will also counteract the harmful effects of bisulfite when added subsequently to dough.

Acetaldehyde has been shown to cause browning of bovine serum albumin solutions at a significantly faster rate than glucose [Mohammed, Olcott, and Fraenkel-Conrat *(12)*]. The action is apparently through cross-linking with the amino group.

Formaldehyde prevents browning probably because of linkage with primary and secondary amines to yield condensation products *(4)*. Compounds of this nature have been isolated. Formaldehyde will also react with a sulfhydryl group (as in cysteine) to form analogs of acetals or hemiacetals. These condensation products are relatively stable.

EXPERIMENTAL

Materials and methods. The semolina used for the processing of the discs was a composite of standard varieties of durum wheat which were grown at 6 locations in North Dakota in 1951. Milling methods have been described by Harris and Sibbitt *(6)*.

The equipment and technique used for processing and drying the discs have been described by Fifield, Smith, and Hayes *(3)* and Smith *et al. (16)*. The Chapman and McFarlane method *(1)* as modified by Hlynka and Bass *(9)* was employed to estimate the reducing groups in the dried discs. A Beckman Model B spectrophotometer equipped with a reflectance attachment was employed to measure objectively color differences (light reflection) in the ground discs. Calcium carbonate was used as a reflectance standard.

The oven-dried discs were heated at 130°C. for one hour, then air dried for 48 hours. Grinding was in a Labconco set to grind material to pass through a 100-mesh sieve. Free amino groups were ascertained by the formol titration employing the modification of Olcott, Saperstein, and Blish *(13)*. The end point of the titration was pH 8.5 as determined with a Beckman pH meter.

RESULTS AND DISCUSSION

Effect of Heat and Pressure. Figure 2 shows the effect on color of heating the pressed disc (3,000 p.s.i. pressure) and tubular macaroni. Pressure is essential in producing the browning, because cut discs with no pressure applied remained an opaque white even after oven drying.

Figure 3 illustrates the changes in reducing groups and light reflectance that take place at different drying temperatures, using a constant press pressure. The effect of temperature is quite marked, the greatest change occurring at 130°C. This temperature was employed for oven drying in all the following work.

Table 1 shows the effect of pressure on the formation of reducing groups and on light absorption. The latter represents the difference between the absorption of the

TABLE 1

Effect of heat, pressure, and glucose on reducing groups[b] and light absorption[c]

Pressure	No glucose			Glucose added (0.5%)			Increase by heat +glucose	Light absorption
	Oven dried	Air dried	Increase	Oven dried	Air dried	Increase		
lbs./sq. in.								%
0	1.54	0.56	0.98	3.78	0.73	3.05	2.07	13.5
1,000	1.93	0.59	1.34	3.95	0.71	3.24	1.90	16.3
2,000	2.91	0.50	2.41	5.14	0.76	4.38	1.97	28.5
3,000	3.14	0.54	2.60	5.24	0.69	4.55	1.95	38.0
4,000	3.17	0.56	2.61	5.32	0.65	4.67	2.06	37.5
5,000	3.21	0.55	2.66	5.33	0.65	4.68	2.02	37.0
6,000	3.21	0.56	2.65	5.33	0.65	4.68	2.03	37.0

[b] Expressed as moles $K_4Fe(CN)_6 \times 10^{-5}$/gm. (dry basis) in all tables.
[c] For discs with added glucose, and represents difference between air- and oven-dried discs in this and following tables.

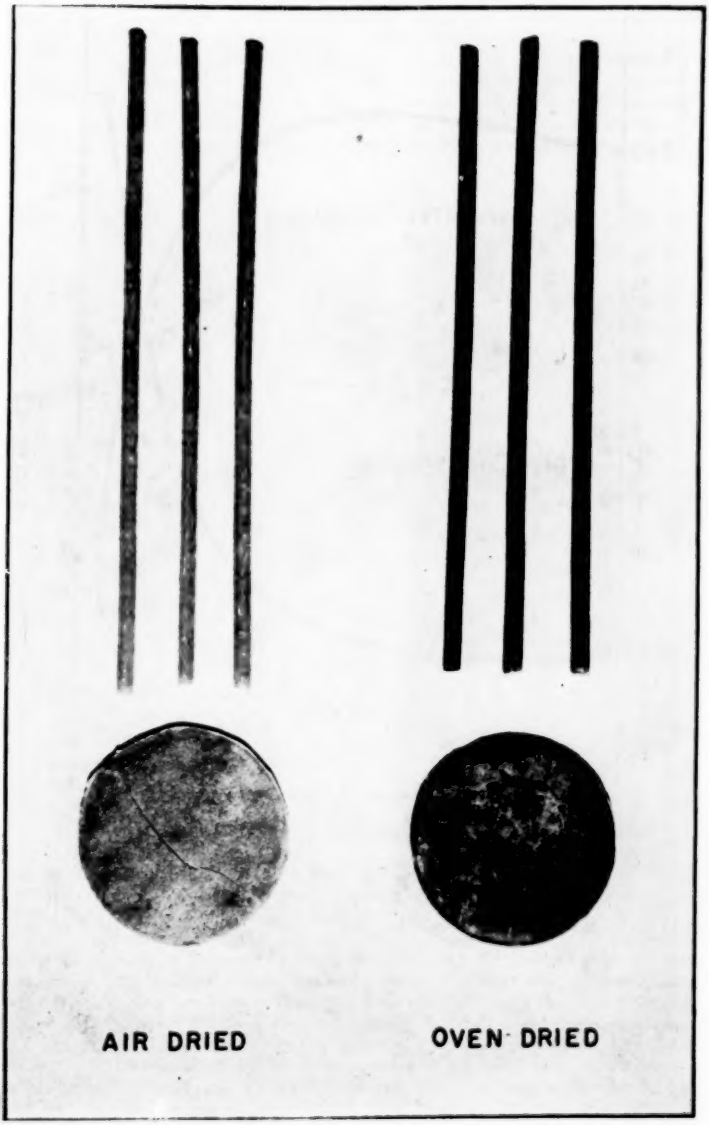

Figure 2. Effect of heating at 130°C. for one hour on the color of tubular and disc macaroni. Discs pressed at 3,000 lb. per sq. in.

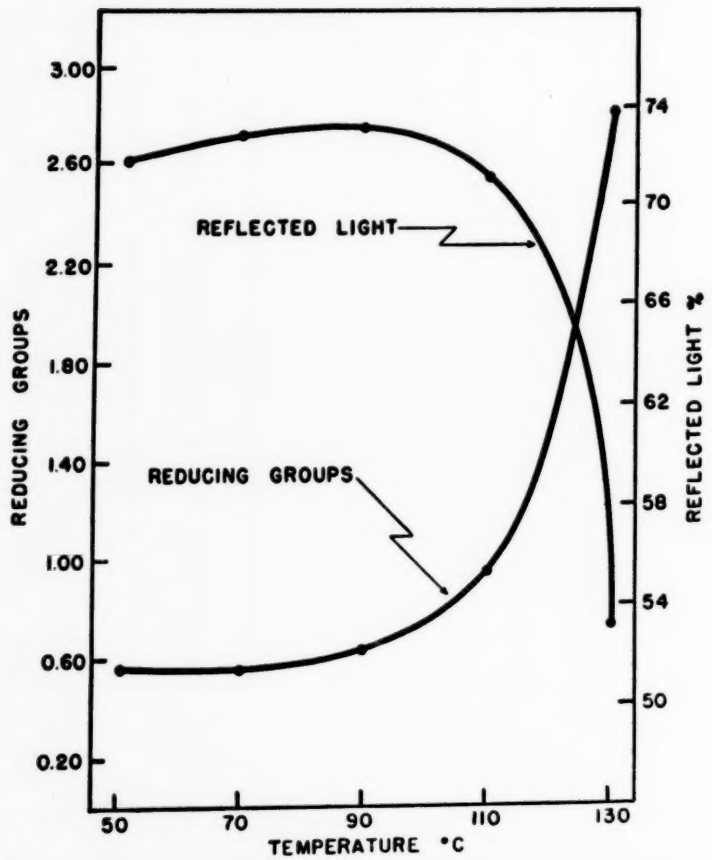

Figure 3. Influence of drying for one hour at different temperatures on reducing groups and reflected light. Reducing groups expressed as moles $K_4Fe(CN)_6 \times 10^{-5}$/gms.

air- and oven-dried discs. Changes in light absorption caused by variations in ingredients and technique are thus eliminated from the values shown. The reducing values doubled with increasing pressure up to 3,000 p.s.i. and then remained constant with additional pressure; therefore this pressure was used in later work. This is also the approximate pressure at which moisture loss markedly decreased (Figure 1). The values for the air-dried samples were the same as those of the semolina (0.58) so mixing, kneading, pressing, and drying had no effect. Hlynka and Bass *(9)* obtained similar results with hard red spring wheat flour where wetting and air drying for 24 hours caused no change in reducing action.

Table 1 also shows the effect of additions of 0.5% of glucose. As found by others *(8, 9)*, glucose increased the formation of reducing groups. The air-dried values were slightly higher than those without glucose so apparently some glucose-protein combination was taking place during air drying. By subtracting the corresponding pressure values without glucose from the discs containing the carbohydrate, it was found

that glucose effected an average increase of 2.00 × 10^{-5} moles of $K_4Fe(CN)_6$ per g. of material. Light absorption values increased to 3,000 lbs. pressure, as did reducing groups, then remained approximately constant. Figure 4 shows the progressive browning or darkening of these discs caused by increasing pressure and concentration of glucose. There was little change in color beyond 3,000 lbs. pressure. Glucose increased color production even in the unpressed discs. In the pressed discs 0.1% glucose caused little appreciable change. The whitish appearance of the 3,000-lb. disc is due to a lighter surface on the upper portion, probably produced by the pressing operation.

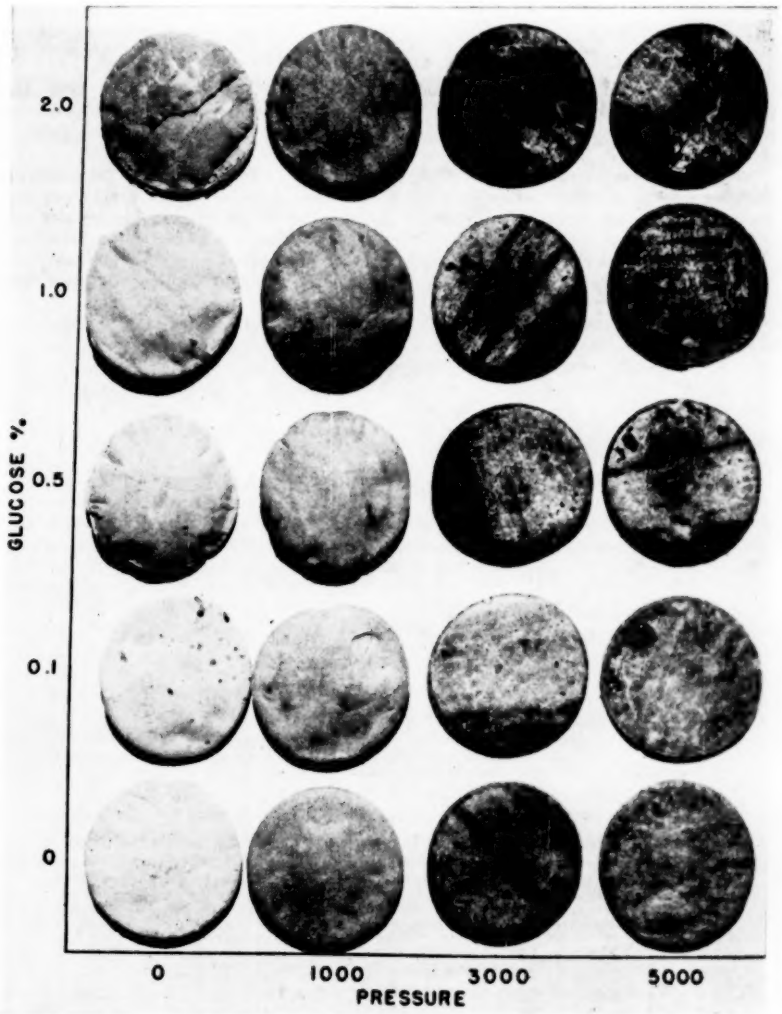

Figure 4. Effect of pressure and glucose concentration on the color of heated macaroni discs.

The effect of pressure on free amino groups in air-dried discs is shown below (on dry basis):

Material	Pressure	Amino nitrogen
	lbs./sq. in.	*mg./10 g.*
Semolina	0	9.5
Ground disc	0	9.2
Ground disc	1,000	12.9
Ground disc	2,000	12.7
Ground disc	3,000	11.8
Ground disc	6,000	11.4

Pressure caused an approximate increase of 38% in amino groups at 1,000 and 2,000 p.s.i. and 26% at 3,000 and 6,000 lbs. As oven drying reduced the solubility of the protein a direct comparison of the amino nitrogen changes was impossible between air- and oven-dried.

Effect of acetaldehyde. Table 2 shows that acetaldehyde increased formation of reducing groups and light absorption after an initial decrease at 0.3% aldehyde concentration. The effect on reducing groups in the absence of glucose was particularly striking at 1.5% of the reagent. Light absorption paralleled reducing group formation. Acetaldehyde in the presence of glucose had the greatest effect at 0.9%. The maximum color, however, was developed at 1.2% of aldehyde. Light absorption increased with acetaldehyde concentration except at 1.5% with glucose.

Effect of carbohydrates. Wheat and its milled products contain small quantities of sugars and starch degradation products. The principal ones are sucrose, glucose (at least in the dough), maltose, and dextrin. Since the browning reaction develops without the aid of added ingredients, it appeared desirable to determine the carbohydrate responsible. Table 3 summarizes this data. The blanks (air-dried discs) with sucrose, maltose, and dextrin showed no effect of increasing concentration, while the values for glucose increased slightly (0.64 to 0.87). The values shown are the oven-dried minus the air-dried disc results.

TABLE 2

The influence of acetaldehyde on reducing groups[d] and light absorption

Acetaldehyde concentration	No glucose		Glucose added (0.5%)		Increase by glucose	
	Red. gr.	Light abs.	Red. gr.	Light abs.	Red. gr.	Light abs.
%	*Red. gr.*	%	*Red. gr.*	%		%
0	2.04	15.5	3.30	26.5	1.26	11.0
0.3	1.95	10.5	4.18	30.0	2.23	19.5
0.6	2.45	16.5	4.65	33.5	2.20	17.0
0.9	2.60	17.5	6.60	35.5	4.00	18.0
1.2	2.97	24.0	6.42	47.0	3.45	23.0
1.5	4.66	31.5	6.53	45.0	1.87	13.5

[d] Values shown are the oven- less air-dried results.

TABLE 3

Effect of added carbohydrates on reducing groups and light absorption

Concentration, %	Sucrose		Dextrin		Maltose		Glucose	
	Reducing groups	Light abs.,%	Reducing groups	Light abs.,%	Reducing groups	Light abs.,%	Reducing groups	Light abs.,%
0.0	2.23	13.0	2.27	7.5	2.21	10.5	2.29	12.0
0.1	2.50	24.0	2.59	16.0	2.26	12.5	2.65	29.0
0.5	2.85	22.7	3.29	24.5	3.33	22.0	4.19	36.5
1.0	3.01	22.7	3.25	23.0	3.74	27.0	6.31	45.0
1.5	2.93	21.5	3.52	28.5	4.21	30.5	6.92	45.0
2.0	2.88	20.7	3.76	29.0	4.40	33.5	7.66	45.0

Sucrose had little if any effect. The method of preparation by acid hydrolysis with possible resultant hydrolysis into glucose or free carbonyl groups might account for the effect of dextrin. Maltose showed somewhat more effect than dextrin. Glucose was the most active. The light absorption values in general corresponded to the reducing group results.

Effect of anti-browning agents. Thirteen percent sodium bisulfite at room temperature has been employed to sever the disulphide linkage in wool [Elsworth and Phillips *(2)*] forming a free thiol group (R–SH). Therefore, the effect of this substance on reducing values was next investigated (Table 4). Any effect of the bisulfite in reducing

TABLE 4

Effect of sodium bisulfite on reducing groups and light absorption

Concentration sodium bisulfite	No glucose				Glucose added (0.5%)				Increase by heat and glucose
	Oven dried	Air dried	Increase	Light abs.	Oven dried	Air dried	Increase	Light abs.	
mg.%	*Reducing groups*			*%*	*Reducing groups*			*%*	*Red. gr.*
0	3.06	0.61	2.45	10.5	4.93	0.75	4.18	22.5	1.73
20	3.19	0.76	2.43	13.0	6.03	0.75	5.28	30.0	2.85
40	3.64	0.84	2.80	17.0	6.28	0.75	5.53	31.3	2.73
60	4.13	0.92	3.21	19.5	6.54	1.06	5.48	35.0	2.27
80	4.30	0.95	3.35	24.3	6.79	1.20	5.59	37.8	2.24

the ferricyanide would be included in the blank (air-dried). Higher concentrations than those used caused too much interference with the Chapman-McFarland method and produced difficulty in processing because of slack, sticky dough. It was found that sodium bisulfite in the concentrations employed increased slightly the number of reducing groups and light absorption. Thus, it did not function as an anti-browning agent in the drying of macaroni discs, possible because of interfering effects of pressure and heat.

There was a linear relation between bisulfite concentration and reducing value for the air-dried discs. Apparently bisulfite had a slight reducing action on the ferricyanide used in the determination of reducing groups. A maximum number of reducing groups was obtained at 20 mg. % bisulfite in the presence of glucose, followed by a slight reduction. The addition of bisulfite may have caused a reduction of cystine to cysteine producing reactive –SH groups *(2, 5, 17)*.

Cysteine hydrochloride has been employed to prevent browning. Since the method used for determining reducing groups was standardized with this amino acid, a study of its effect on browning seemed worthwhile. When an aqueous solution of cysteine hydrochloride or of glucose was evaporated to dryness no color change was observed, but when they were mixed together and dried a deep brown color developed. Maillard *(10)*, earliest of the investigators of browning, noted that reducing sugars and amino acids reacted to yield a colored product which he termed melanoidin.

TABLE 5

Effect of cysteine hydrochloride on reducing groups and light absorption

Cysteine hydrochloride Moles × 10^{-4}	Glucose* added (0.5%)	Light absorbed	No glucose*	Light absorbed	Increase by glucose
	Red. gr.	*%*	*Red. gr.*	*%*	*Red. gr.*
0	4.11	23.0	2.33	11.8	1.78
0.4	4.26	34.3	2.91	16.7	1.35
0.8	4.81	39.5	3.21	17.7	1.60
1.2	5.93	36.0	3.35	24.2	2.58
1.6	6.66	37.0	3.51	21.7	3.15
2.0	6.82	40.5	3.48	27.2	3.34

*Values shown are the oven- less air-dried results.

Table 5 summarizes the effect of cysteine on the formation of reducing groups. Cysteine had no effect on the air-dried discs. However, the pressed discs showed that this substance increased the number of reducing groups until a concentration of 1.6 $\times 10^{-6}$ moles was attained. As the amount of naturally occurring carbohydrate available might have been the limiting factor, 0.5% of glucose was added to the mixes. The results showed a marked increase in reducing groups, demonstrating that the amino acid and the carbohydrate were the principal reactants.

Cysteine hydrochloride and glucose will combine in equimolar quantities in neutral solution forming a compound that has a melting point with decomposition at 167°C. The compound was prepared by the method proposed by Schubert *(15)* and tested for reducing groups. Table 6 shows that cysteine was most effective if added as a complex

TABLE 6

Influence of the cysteine hydrochloride-glucose complex on reducing groups

Complex concentration	Oven dried	Air dried	Increase by heating
%			
0	2.36	0.51	1.85
0.05	3.49	1.10	2.39
0.10	5.85	1.87	3.98
0.15	6.11	2.60	3.51
0.20	6.99	3.15	3.84
0.25	7.60	3.97	3.63

with glucose. The 2.0 $\times 10^{-6}$ moles concentration of the amino acid (Table 5) corresponds approximately to 0.02 mg. % which is 25 times less than the smallest amount used of the glucose-cysteine compound. This suggests that more than a single amino acid and carbohydrate are involved. The air-dried values showed that the reducing action of the compound was linear over the concentration range studied in the absence of pressure. Increase in reducing groups caused by heat reached a maximum at 0.1% concentration.

Effect of enzymatic activity. Preliminary experiments showed that incubation for 24 hours in a moist atmosphere increased browning in oved-dried discs. To determine possible effects of amylolytic or proteolytic activity, purified malt diastase and papain were added in various concentrations to the mix, and the reducing values of the discs determined as before. The data are presented in Table 7.

Small concentrations of both enzymes increased the formation of reducing groups and light absorption to approximately the same extent. The addition of glucose (0.5%) with papain gave an average increase of 1.42 $\times 10^{-5}$ moles of $K_4Fe(CN)_6$, merely an additive effect. Glucose also increased light absorption values.

TABLE 7

Effect of enzymatic activity on reducing groups and light absorption

Malt diastase[1]			Papain[1]					
Concentration, %	No glucose		Concentration, %	Glucose added, 0.5%		No glucose		Inc. by glucose	
	Reducing groups	Light abs.,%		Reducing groups	Light abs.,%	Reducing groups	Light abs.,%	Reducing groups	Light abs.,%
0	2.42	8.5	0	3.99	24.0	2.31	8.5	1.68	15.5
0.04	2.84	16.3	0.02	4.25	26.5	3.03	16.5	1.22	10.0
0.08	3.10	20.0	0.04	4.77	37.8	3.49	21.3	1.28	16.5
0.16	3.41	20.0	0.06	4.97	41.0	3.46	20.2	1.51	20.8
0.32	3.85	26.5	0.08	5.15	39.2	3.75	24.0	1.40	15.2

[1] Values shown are the oven-less air-dried results.

Effect of formaldehyde. As has been pointed out formaldehyde prevents the occurrence of browning. To ascertain whether it would inhibit the formation of reducing groups in macaroni discs, a semolina dough containing 3% formaldehyde was air dried for 48 hours and ground, then remixed and pressed. The following results were obtained:

	Moles $K_4Fe(CN)_6$ $\times 10^{-5}$/gm.
Semolina	0.22
Air-dried discs	0.21
Oven-dried discs	0.17
Air-dried dics, 0.5% glucose added	0.17
Oven-dried discs, 0.5% glucose added	0.15

The formaldehyde evidently tied up the 2 reactive groups (-SH, $-NH_2$) of the protein. Identity of the group involved remains for future inquiry. The results from other workers *(11, 12)* and the present findings incline to the amino group. Alkaline dispersions of all the ground discs in this study showed no evidence of free -SH, as determined by the nitro-prusside test.

Correlation of disc color with reducing values. Apparently there is a relationship between color and reducing groups in oven-dried discs. A correlation of —0.909 was obtained between reducing groups and % light reflected for 95 samples. Since there appeared to be variations of color in the air-dried discs due to treatment, the change in reflected light (light absorption) between air- and oven-dried discs was correlated with the corresponding differences in reducing groups; a coefficient of +0.926 was obtained. Figure 5 is a scatter diagram which represents this relationship. The close relation between the two series of values is very marked.

SUMMARY AND CONCLUSIONS

Heating at 130°C. caused the formation of a characteristic brown color in pressed macaroni discs. The depth of color was directly related to the degree of pressure below 3,000 p.s.i. Above this pressure, the effect of pressure increases became less noticeable. Drying at lower temperatures decreased color formation. Heat alone without pressing failed to induce browning.

Pressure also increased reducing groups in the dried discs as well as the light absorption. Addition of glucose had the greatest effect, while the effects of maltose, dextrin, and sucrose decreased in the above order. Acetaldehyde also increased the formation of reducing groups and light absorption. Sodium bisulfite, although known in some instances to inhibit the production of browning, increased to some extent reducing groups and light absorption. Cysteine hydrochloride, particularly when combined with glucose, exerted a favorable influence on these reactions. Formaldehyde, as would be expected from a knowledge of the literature, prevented the reactions, apparently by combining with reactive groups of the protein. The identity of the group was not clearly established, although the evidence tends to indicate amino groups.

Both proteolytic and amylolytic enzymes increased reducing groups and light absorption.

Color production in pressed macaroni during oven drying is apparently related to the formation of reducing compounds between amino acids and carbohydrates, possibly through a linkage with the amino groups. An alternative linkage might be through sulfhydryl groups of the amino acid.

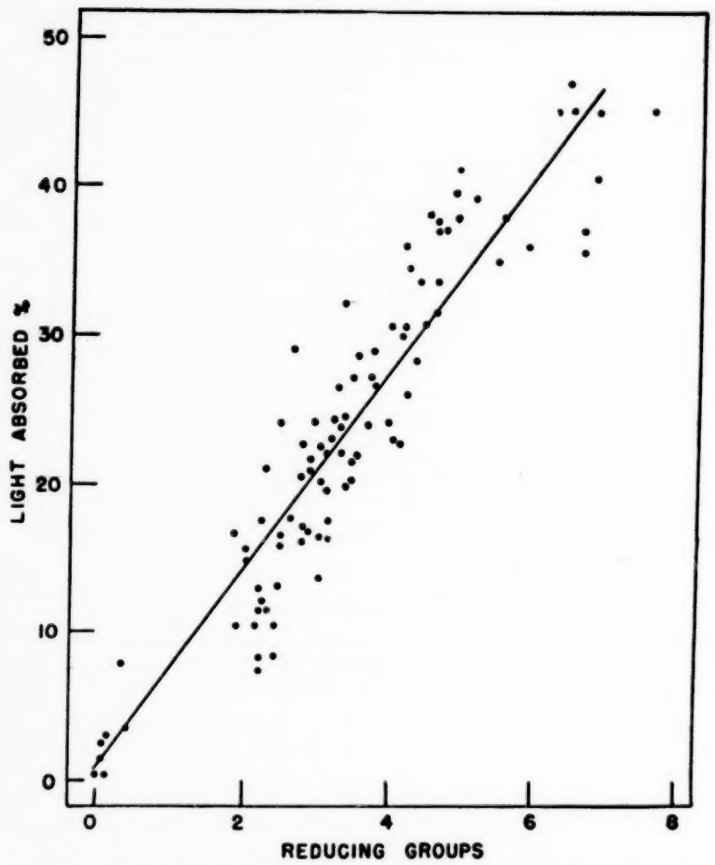

Figure 5. Scatter diagram of reducing groups and light absorption values of macaroni for 95 discs. Regression of light absorption on reducing groups is also shown.

Acknowledgment

The authors wish to acknowledge the interest and advice of Dr. Glenn Smith, Chief of Plant Industry at this Station, in this project. The technical assistance of G. M. Scott is also acknowledged.

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SURVIVAL OF STAPHYLOCOCCI AND SALMONELLAE IN PUDDINGS AND CUSTARDS PREPARED WITH EXPERIMENTALLY INOCULATED DRIED EGG[a]

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Because Salmonellae have been found in dried eggs, extensive limitation has been placed on the use of this product in many foods, including puddings *(7)*. The research reported herein is concerned with the effect of the application of heat in the absence of added acid in destroying micro-organisms inoculated into eggs used in puddings and custards. This is the second part of a bacteriological study on foods prepared with dried eggs; the first part of the study (on cooked salad dressing) has already been reported by Kintner and Mangel *(6)*.

Catheart, Godkin, and Barnett *(1)* concluded from a study of various pastry fillings that the acidity of fruit filling (made with water) checked the growth of Staphylococcus. However, they found the use of milk in cream fillings supported the growth of this type of bacteria. Catheart, Merz, and Ryberg *(3)* reported that the commercial procedure of bringing cream fillings to a boil after the addition of the egg and starch mixture, rendered the contaminated filling free from *Staphylococcus aureus* and *Salmonella enteritidis*. The fillings were tested immediately after cooking.

A large number of food poisoning outbreaks, traced to Staphylococcus contaminated cream filled pastries *(4)*, have indicated that in addition to proper heating, prompt cooling and refrigeration are necessary. Dack *(5)* infers the necessity of refrigeration when he states that 5 to 12 hours at a suitable temperature, provide an environment for the production of Staphylococcus enterotoxin which is responsible for the majority of bacterial food poisoning outbreaks.

The present study was made to check the influence of temperature and sugar concentration on the survival of both Staphylococci and Salmonellae experimentally inoculated into dried eggs used in preparing custards and puddings. The prepared dishes were held under aseptic conditions for varying periods of time to see whether viable micro-organisms remained after storage at either room or refrigerator temperature.

EXPERIMENTAL

The dried eggs used in this part of the study were from the same lot as those described in the previous study *(6)*. The seven species of Salmonella[b] were: *Salmonella typhimurium*, *Salmonella enteritidis*, *Salmonella pullorum* No. 19, *Salmonella gallinarum*,

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[b]Cultures of bacteria used were obtained from the Bacteriology Department of the University of Missouri Medical School and from Dr. Philip Edwards of the Public Health Service, Chamblee, Georgia.

Salmonella choleraesuis, *Salmonella orienburg*, and *Salmonella anatum*. A food poisoning strain of *Micrococcus pyogenes* var. *aureus* (*Staphylococcus aureus*) was also used.

Basic formulae. In order to obtain vanilla, butterscotch, and chocolate puddings which were typical in flavor and texture, various recipes were tested until a good basic formula for each type of product was obtained. The dried eggs were reconstituted, using 12 g. with 30 ml. of water as equivalent to one fresh egg, and were used within fifteen minutes after reconstitution. Temperatures were recorded for the starch, sugar, milk mixture after cooking 12 minutes, then the egg suspensions were added and the time and temperatures were recorded as soon as the mixture had thickened. In the vanilla puddings, the sugar was increased to produce products of 18 and 25% sugar concentration. The basic custard recipe was employed for both the stirred and baked custards. For the stirred custard the cold ingredients were combined and cooked over boiling water. The length of cooking time was recorded. For baked custard, the cold ingredients were combined and baked at 325°F. The formulae used as bases for preparation of the experimental puddings and custards are reported in Table 1.

TABLE 1

Basic formulae

	Dried egg	Water	Milk	Sugar	Salt	Vanilla	Cornstarch	Brown sugar	Butter	Chocolate
	g.	*ml.*	*ml.*	*g.*	*g.*	*ml.*	*g.*	*g.*	*g.*	*g.*
Vanilla pudding										
12% sugar	12	30	488	66	1	4	16			
18% sugar	12	30	488	100	1	4	16			
25% sugar	12	30	488	150	1	4	16			
Chocolate pudding	12	30	488	100	1	4	16			28
Butterscotch "	12	30	610		1	4	24	132	13	
Custard										
Stirred	24	60	488	50	0.5	4				
Baked	24	60	488	50	0.5	4				

Procedure. The puddings and custards were prepared using proportions and quantities of the basic formulae, substituting 15 ml. of broth-bacteria culture for 15 ml. of water in the reconstitution of each 12 g. of dried egg. The starch mixture of the pudding was cooked in the top of the double boiler for 12 minutes, to a temperature of 92-98°C. The reconstituted dried egg was then added and cooked for one-half minute, until a temperature of 78-86°C. was obtained. The stirred custards were cooked in the top of a double boiler from 5 to 6 minutes to a temperature of 91-93°C. The custards were baked for 45 minutes at 325°F. At this stage an internal temperature of approximately 91°C. was reached. All cooked puddings and custards were held at room temperature for varying times. Duplicate samples of some of the puddings were held refrigerated.

Media and method of plating. As soon as the puddings and custards were made, samples were removed and placed in sterilized flasks. Transfers were made immediately and after 1, 2, 3, 4, and 24 hours from the products held at room temperature and after 24 hours from those held at refrigerator temperature. Bacto-Tryptose agar (Difco) plates were used to check for growth of Staphylococci. SS (Shigella-Salmonella) agar (Difco) plates were used to check for possible Salmonella growth. All plates were incubated at 37°C. for 24 hours. If bacterial colonies were present, they were identified by growth characteristics. If growth occurred on the SS plates, representative colonies were transferred to Kligler's iron agar (Difco) slants to further indicate the possible presence of Salmonella.

RESULTS AND DISCUSSION

The viability of the bacteria in the puddings and custards prepared with experimentally inoculated dried eggs is recorded in Table 2. The chocolate pudding (containing 16% sugar) showed growth of *Staphylo-*

TABLE 2

Viability of bacteria inoculated into dried eggs used in preparing puddings and custards

Test organisms	Hours held at room temperature	Plate growth of						
		Pudding					Custard	
		Vanilla			Chocolate	Butterscotch		
		12% sugar	18% sugar	25% sugar			Stirred	Baked
		6.59 pH	6.64 pH	6.64 pH	6.39 pH	6.40 pH	6.71 pH	
Staphylococcus aureus	0	−	−	−	−	−	−	−
	1	−	−	−	−	−	−	−
	2	−	−	−	−	−	−	−
	3	−	−	−	−	−	−	−
	4	−	−	−	+	−	−	−
	24	−	−	−	+	−	−	−
Salmonella typhimurium	0	−	−	−	−	−	−	−
	1	−	−	−	−	−	−	−
	2	−	−	−	−	−	−	−
	3	−	−	−	−	−	−	−
	4	−	−	−	−	−	−	−
	24	−	−	−	−	−	−	−
Salmonella enteritidis	0	−	−	−	−	−	−	−
	1	−	−	−	−	−	−	−
	2	−	−	−	−	−	−	−
	3	−	−	−	−	−	−	−
	4	−	−	−	−	−	−	−
	24	−	+	−	−	−	−	−
Salmonella gallinarum	0	−	−	−	−	−	−	−
	1	−	−	−	−	−	−	−
	2	−	−	−	−	−	−	−
	3	−	−	−	−	−	−	−
	4	−	−	−	−	−	−	−
	24	−	−	−	−	−	−	−
Salmonella pullorum	0	−	−	−	−	−	−	−
	1	−	−	−	−	−	−	−
	2	−	−	−	−	−	−	−
No. 19	3	−	−	−	−	−	−	−
	4	−	−	−	−	−	−	−
	24	+	−	−	−	−	−	−
Salmonella choleraesuis	0	−	−	−	−	−	−	−
	1	−	−	−	−	−	−	−
	2	−	−	−	−	−	−	−
	3	−	−	−	−	−	−	−
	4	−	−	−	−	−	−	−
	24	−	−	−	−	−	−	−
Salmonella orienburg	0	−	−	−	−	−	−	−
	1	−	−	−	−	−	−	−
	2	−	−	−	−	−	−	−
	3	−	−	−	−	−	−	−
	4	−	−	−	−	−	−	−
	24	+	−	−	−	−	−	−
Salmonella anatum	0	−	−	−	−	−	−	−
	1	−	−	−	−	−	−	−
	2	−	−	−	−	−	−	−
	3	−	−	−	−	−	−	−
	4	−	−	−	−	−	−	−
	24	−	−	−	−	−	−	−
Control	0	−	−	−	−	−	−	−
	1	−	−	−	−	−	−	−
	2	−	−	−	−	−	−	−
	3	−	−	−	−	−	−	−
	4	−	−	−	−	−	−	−
	24	−	−	−	−	−	−	−

coccus aureus after being held for 4 as well as after 24 hours at room temperature. Growth of *Salmonella pullorum* and *Salmonella orienburg* occurred in puddings made using the 12% sugar-vanilla formulae, and growth of *Salmonella enteritidis* occurred in an 18% sugar-vanilla pudding after holding for 24 hours at room temperature. No growth occurred in these puddings with shorter storage periods. None of the 25% sugar-vanilla puddings or butterscotch puddings (18% sugar) showed viable bacteria after even 24 hours' storage at room temperature. No bacterial growth occurred in any of the puddings held for 24 hours at refrigerator temperature.

Catheart and Merz *(2)* reported that in chocolate and cocoa puddings, growth of *Staphylococcus aureus* was inhibited, due to a lowered pH. Although the chocolate puddings prepared in this study had lower pH's, with a mean of 6.39, than the butterscotch and vanilla puddings, no inhibiting effect was observed. It is postulated that the use of a formulae relatively high in milk and eggs provides a medium in which the micro-organisms are more resistant to the effect of the acidity of chocolate. Tanner has stated *(8)* that sugar does not exert a preservative effect until the concentration reaches 25 g. in 100 ml. Since only one pudding in this series contained as much as 25% sugar, no conclusions as to the preservative effect of sugar in puddings are drawn from this study.

Apparently, under the conditions of this experiment, the heat treatment used in the preparation of the puddings was effective in reducing the number of micro-organisms to a minimum. No evidence of growth of the organisms was obtained in 3 hours or less at room temperature. The identification of the inoculated micro-organisms in 4 puddings held for longer periods at room temperatures demonstrates that these products, unlike the acid salad dressings, provide a favorable medium for growth.

There was no variation in the formula used in the stirred and baked custards. At the rate of heating used in this experiment, one cup of stirred custard made from dried eggs was just thickened in 5 to 6 minutes. The temperature reached at the thickening point for both stirred and baked custards was 91-93°C. The thickening temperatures at other rates of heating were not determined. Findings of this experiment suggest that the temperature used to thicken the dried eggs in custards was sufficient to destroy all pathogenic bacteria inoculated into the dried eggs.

Results of this experiment seem to indicate that the recommendation for the limitation placed by the U.S.D.A. *Bulletin* No. 136 *(7)* on the use of dried eggs in cream puddings and fillings and soft custards might well be given further consideration.

No bacterial growth occurred in any of the baked custards which had been inoculated before cooking. The results of this study on baked custard supported the previous report *(7)* that this is a safe way to use dried eggs.

CONCLUSIONS

Minimum temperature for thickening egg, 78-86°C., does not destroy all bacteria inoculated into the dried egg in these puddings. However, under the conditions of this experiment the number of micro-organisms was reduced to a minimum and the growth was inhibited in all cases for at least

3 hours. These puddings, like all other cream puddings or fillings may be potentially dangerous if contaminated egg, either fresh, frozen or dried, is used. Prompt refrigeration minimizes this danger.

At the temperature, 91-93°C., used in the preparation of the stirred and baked custards in this experiment, none of the inoculated bacteria remained viable.

Danger of food poisoning from the use of contaminated eggs in such products as puddings and custards would appear to be remote if these products are adequately heated and refrigerated. It is particularly important to avoid contamination after cooking since these products, unlike the acid salad dressings, do provide a favorable medium for bacterial growth.

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A COMPARISON OF THE RETENTION OF VITAMIN A IN MARGARINES AND IN BUTTERS BASED UPON BIOASSAYS

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Vitamin A is quite resistant to destruction, especially when stored under such inert gases as nitrogen or carbon dioxide. In the presence of air, vitamin A is oxidized to biologically inactive compounds; this rate of breakdown is accelerated when the air is bubbled through the vitamin A-containing solution. The speed of destruction is also increased by augmented temperatures. Vitamin A can likewise be destroyed by exposure of its solution to ultraviolet light. In fact, one method for the estimation of the vitamin A content of fats involves the determination of the extent of absorption at 328 mμ before and after exposure of the test solution to ultraviolet light having the wave-length *(8)*; the resulting difference in extinction value represents the proportion of the absorption attributable to vitamin A.

Studies on the resistance of vitamin A to destruction during storage in the case of fats are quite limited. Brence and Nelson *(2)* reported only slight changes in the vitamin A and carotene values of butters stored for 75 or 180 days at 0°F.(—18°C.) or for 30 or 60 days at 40°F.(4°C.). Murray and co-workers *(7)* noted that the average vitamin A content of margarine samples produced by the 10 manufacturers supplying 97.5% of the total margarine in Canada was 4,062 I.U./100 g. (18,500 I.U./lb.). The above samples were obtained from retail sources, and represented varying periods of storage. In a recent study carried out by Melnick *et al. (6)*, it was shown that both vitamin A and carotene are remarkably stable (97-98% retention) in margarine when stored at 45°F.(7°C.) for a period blanketing the shelf life of the product. A somewhat increased rate of destruction was recorded when the margarines were stored at 75°F.(24°C.) in which a retention of more than 90% was found after one month. In all of the above tests, physicochemical methods of proven reliability *(4, 5)* were employed for the estimation of the vitamin A and carotene.

In the present report, a study was made of the speed at which vitamin A is lost from margarines and from butters, as affected by storage under temperatures favorable for the retention of vitamin A, or in an environment which might be expected to be decidedly unfavorable for the preservation of the fat-soluble vitamins. In these studies, the results were obtained by bioassay procedures.

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EXPERIMENTAL

The investigations of vitamin A retention in margarines and butters were made on samples of the products of different margarine manufacturers. They were picked up from the regular production lines by disinterested individuals and were shipped by express to the laboratory. The samples were thereafter listed under a code letter known only to the investigators, and the results are so reported in the present communication. The following brands are included in the studies: Armour and Co. (Mayflower); The Best Foods, Inc. (Nucoa); Cudahy Packing Co. (Delrich); Jelke Co. (Good Luck); Kraft Foods Co. (Parkay); Shedd-Bartush Co. (Keyko); Standard Brands (Blue Bonnet); and Swift and Co. (Allsweet). In each case the authors were informed as to what type of vitamin A was being used at the time at which the samples were obtained.

Three of the butter samples were obtained from a creamery operated at LaCrosse, Wisconsin, by Swift and Co. Samples 9 and 10 were from predominantly Holstein herds, and were low in carotene, while sample 23 was from a Guernsey herd, and was high in carotene. Sample 24 was a California butter churned locally. Artificial coloring was not present in any samples. The butters were obtained at a time when the cows were on green pasture. The samples from Wisconsin were forwarded to the laboratory in sealed, iced containers.

Samples of margarine and butter, suitably labeled, were stored in their original wrappers as follows: (a) in a commercial cold-storage warehouse at approximately 14°F.(−10°C.) for 2 years; (b) at the Hancock Foundation, in a cold room which was maintained at approximately 41°F.(5°C.); (c) in an incubator kept at 64°F.(18°C.); and (d) in a heated room which had a temperature of approximately 82°F.(28°C.) during the day, with somewhat lower temperatures during the night. The latter samples were stored for various periods before bioassay in a cold room at 41°F.(5°C.).

It was the original plan of the investigation to follow the change in the vitamin A content of the several samples at frequent intervals by spectrophotometric assay, making spot checks by means of bioassay. For these chemical tests, the floridin technic of Awapara *et al. (1)* was employed. Although satisfactory values could be obtained by this procedure with fresh margarines, no satisfactory correlation was possible for the samples which had been stored over long periods of time, and in which a considerable destruction of the vitamin A had been noted. Because of the uncertainty of the interpretation of these studies, only the results of bioassays are reported here.

The bioassays were carried out by the U.S.P. XIII method, using vitamin A reference cod-liver oil as a standard for the assays carried on during 1946 and 1947, while reference vitamin A acetate was employed for the tests performed in 1948. In the first series of tests with reference cod-liver oil, the standard was fed at 1.5 and 2.5 I.U. per day while, in the last tests, vitamin A acetate was given in daily dosages of 1.25 and 2.25 I.U. Since 2 different samples of reference vitamin A were used for the bioassays, a factor was established by biological assay and used to equate the results of the earlier tests with those obtained on the vitamin A acetate. In some cases multiple-level bioassays were carried out with the test fats. However, in most instances, the results were determined from single-level tests. The dosages of margarines or of butters in these cases were at levels calculated as approximately midway between the dosages of the standards. The fats fed were mixtures of the whole melted product rather than the fat alone. Negative control groups were used in all tests. Calculation of the vitamin A content in the unknown samples was made by the method of Coward *(3)*.

RESULTS AND DISCUSSION

A summary of the bioassays on 8 brands of margarines before and after storage over different periods and at several temperatures is given in Table 1, while Table 2 records similar data for samples of 4 different butters.

The vitamin A content of the original samples of the 8 margarines assayed averaged 42.9 U.S.P. units per g., which exceeds that declared on the label (15,000 U.S.P. units per lb. or 33.0 U.S.P. units per g.) by 9.9 units per g., or by 30%. Seven of the 8 samples had a potency exceeding 33.0 units per g.

TABLE 1

The vitamin A content of different brands of vegetable margarines, as determined by bioassay before and after storage for different periods, at several temperatures

Type of vitamin A used	Sample No.	Vitamin A content in U.S.P. units per g.[c]								
		Original	14°F. 2 years	41°F. 52-60 weeks	64°F.		82°F.			
					17[d] weeks	45[e] weeks	6 weeks	15 weeks	21 weeks	41[f] weeks
Alcohol	1	34.2[g]	37.6	25.2	21.7	14.6	35.0	....	24.3	10.7
			110	*74*	*63*	*43*	*102*	....	*71*	*31*
	2	41.5	39.4	38.2	36.9[g]	29.2	45.0	....	....	21.1
			95	*92*	*89*	*70*	*108*	....	....	*51*
Distilled ester	3	41.5	35.3	34.2	37.0	24.6	....	....	....	11.4
			85	*82*	*89*	*59*	....	....	....	*27*
	4	48.3	43.1	34.0	42.5	30.6	46.0	41.0	42.6	28.0
			89	*70*	*88*	*63*	*95*	*85*	*88*	*58*
	5	53.0	33.4	37.7[g]	38.1	31.8	....	....	....	19.2
			63	*71*	*72*	*60*	....	....	....	*36*
	6	29.5	23.3	18.3	16.9	10.6[g]	....	....	....	< 5.9
			79	*62*	*57*	*36*	....	....	....	*<20*
Natural ester	7	52.4	35.0	47.1	34.4	28.7	....	....	....	13.9
			67	*90*	*66*	*55*	....	....	....	*26*
	8	42.5	36.2	43.2	34.2	17.6	....	34.2	21.8	12.3
			85	*102*	*80*	*41*	....	*80*	*51*	*29*

[c] The figures in italics represent the percentage of vitamin A present as compared with the original value. [d] Plus 35-50 weeks at 41°F. [e] Plus 15-31 weeks at 41°F. [f] Plus 46 weeks at 41°F. [g] Multiple bioassay.

In the samples assayed after being stored for 2 years at 14°F., the average vitamin A was 35.4 units per g. (+7.3% over declared potency); 7 of the 8 samples exceeded that declared on the label. After storage at 41°F. for 52 to 60 weeks, 6 of the 8 samples of margarine had a potency exceeding 33.0 units per g.; the average vitamin A content was 34.7 units per g., or 5% in excess of the declared value.

When the margarines were stored at room temperature for 4 months (17 weeks), the vitamin A exceeded the declared potency in 6 of the 8 brands. After storage under similar conditions for 45 weeks, the vitamin A content was reduced to a low value; however, in 4 of the 8 brands tested, the vitamin A content at this time was still within 13% of the declared potency.

When the margarines were stored at 82°F., all of the 3 samples analyzed after 6 weeks, and both of the 2 samples assayed after 15 weeks, contained vitamin A in excess of their declared potencies. In the samples stored for 21 weeks, only one of the 3 samples had a vitamin A content in excess of the declared potency. Finally, none of the brands subjected to the 82°F. storage for 41 weeks had a vitamin A content of as much as 33 units per g.

The efficiency of retention of vitamin A is more satisfactory in some margarines than in others. For example, vitamin A was retained especially well in samples 2 and 4. On the other hand, in the case of sample 6, not only was the amount of vitamin A lowest in the original material of any brand tested, but it was destroyed to a greater degree than in other brands in all cases in which the product was stored, except in the sample stored at 14°F. for one year.

In the case of the butters, 3 of the 4 samples had a high initial vitamin A content. This was expected since the products were so-called "summer" butter obtained when the cows were in green pasture. The average vitamin A value was initially 55.5 U.S.P. units per g.; the mean value after the 2-year storage period at 14°F. was 44.2 units, and that after storage for 52 to 60 weeks at 41°F. was 47.7 units per g.

In the tests made at 64°F., 2 of the 4 samples exhibited a marked fall in vitamin A after 17 weeks, while 3 of the 4 samples stored for 41 weeks at 64°F. had too low a vitamin A content to be determined at the level tested. The results on the butters subjected to 82°F. were quite similar to those obtained at 64°F.

A better evaluation of the stability, on storage, of vitamin A in margarines, as related to the type of vitamin A used for supplementation, is given by comparison of the percentage changes in potency from the original value. This affords a method for comparison with the data on butter. This information is summarized in Table 3.

No marked differences obtain between the proportion of vitamin A retained after different periods of storage, irrespective of whether the vitamin A supplement was added as the alcohol, as the distilled esters, or as the natural esters. The proportion of vitamin A retained in the butter samples paralleled that in the margarines. However, in the samples assayed after storage at 64°F. for 45 weeks, the results on butters were decidedly inferior to those on margarines. Whereas a mean value of

TABLE 2

The vitamin A content of butters from several sources, as determined by bioassay before and after storage for different periods, at several temperatures

Sample No.	Source	Vitamin A content in U.S.P. units per g.[b]								
		Original	14°F. 2 years	41°F. 52-60 weeks	64°F.		82°F.			
					17[1] weeks	45[1] weeks	6 weeks	15 weeks	21 weeks	41[k] weeks
9	Minnesota	56.0[1]	42.5	54.8	57.2	<22.4	48.0	....	35.7	21.6
			76	*98*	*102*	*<40*	*86*	....	*64*	*39*
10	Wisconsin	61.5	47.5	52.2	53.6	38.3	....	49.5	....	31.7
			77	*85*	*87*	*62*	....	*81*	....	*52*
11	Texas	67.0[1]	53.9	59.2	21.4	<26.8	....	48.5	43.5[1]	<13.4
			80	*88*	*32*	*<40*	....	*72*	*65*	*<20*
12	Oregon	37.5	32.9	24.6	21.7	<15.0	....	....	25.5	8.7
			88	*66*	*58*	*<40*	....	....	*68*	*23*

[b] The figures in italics represent the percentage of vitamin A present as compared with the original value. [1] Plus 35-50 weeks at 41°F. [j] Plus 15-31 weeks at 41°F. [k] Plus 46 weeks at 41°F. [l] Multiple bioassay.

TABLE 3

Summary table of average vitamin A retention in per cent in margarines and butters after storage for different periods at several temperatures

Substance assayed	Per cent of original vitamin A content							
	14°F. 2 years	41°F. 52-60 weeks	64°F.		82°F.			
			17 weeks	45 weeks	6 weeks	15 weeks	21 weeks	41 weeks
Margarines (supplements added):								
Alcohol	102(2)	83(2)	76(2)	56(2)	105(2)	....	71(1)	41(2)
Distilled ester	79(4)	71(4)	78(4)	54(4)	95(1)	85(1)	88(1)	35(4)[m]
Natural ester	76(2)	96(2)	73(2)	48(2)	....	80(1)	51(1)	28(2)
Average, all tests	83(8)	80(8)	76(8)	53(8)	102(3)	82(2)	70(3)	35(8)[m]
Butters	78(4)	84(4)	70(4)	<45(4)[n]	86(1)	76(2)	66(3)	34(4)[m]

Figures in parentheses are number of tests included in the average. [m] Including one value <20% considered as 20%. [n] Including 3 values of <40% considered as 40%.

53% of the original vitamin A was still present in the margarines at that time, a figure considerably under 45% was noted for the butters. Three of the 4 samples of butter had such a low vitamin A content that the gain-in-weight of the rats was too far below that of the animals on the standard to be calculated. In these 3 tests, the vitamin A was simply listed as "less than 40%," it may have approached 0. In arriving at an average of 45%, the figures used in these cases were calculated as 40%.

It should also be noted that the margarines stored for as long as 45 weeks at 64°F., remained sweet, and were found to be appetizing when consumed by members of our laboratory staff. In fact, none of the samples were objectionable in flavor or odor, even after prolonged storage. The butters stored at 64°F. proved to be completely unappetizing after several weeks, due to the development of rancidity.

SUMMARY

1. The vitamin A retention in margarine was, on an average, in excess of 75% of the original content, after storage for 2 years at 14°F.(−10°C.), for 52 to 60 weeks at 41°F.(5°C.), for 17 weeks at 64°F. (18°C.), and for as long as 15 weeks at 82°F.(28°C.).

2. The vitamin A content in the margarines exceeded 15,000 U.S.P. units per lb. (33 units per g.) in the following samples of different brands tested: original samples, 7 of 8; 14°F. (2 years), 7 of 8; 41°F. (52 to 60 weeks), 6 of 8; 64°F. (17 weeks) 6 of 8; 82°F. (6 weeks), 3 of 3; and 82°F. (15 weeks), 2 of 2.

3. No differences were noted in the retention of vitamin A in margarines, irrespective of whether the vitamin added was in the form of the alcohol, the distilled esters, or the natural esters.

4. The average original vitamin A content of the 8 margarines was 42.9 units per g.

5. The original vitamin A levels in the 4 summer butter samples averaged 55.5 U.S.P. units per g.

6. The percentage of vitamin A retention of butters undergoing storage at the several temperatures was usually slightly less than in the margarines. This was particularly true in samples stored at 64°F. for 45 weeks.

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THE RETENTION OF PREFORMED VITAMIN A AND CAROTENE IN MARGARINE BASED UPON PHYSICO-CHEMICAL ASSAYS

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Deuel and Greenberg *(2)* have reported on the comparative stability of vitamin A in margarines and in summer butters based upon bioassay findings. These studies involved holding the products at various temperatures for extended periods of time. The conclusion was justified that vitamin A retention in margarine was highly satisfactory, being somewhat better than that noted in comparable tests conducted on the summer butters.

The long holding periods of one to 2 years at refrigeration temperatures are of practical value in the case of butters but of only academic interest insofar as margarines are concerned. Margarines are seldom of more than 3 months of age, since supply is adjustable to demand for the product. The Deuel-Greenberg study was of necessity limited to a relatively small number of samples, 8 margarines and 4 butters. Biological assays are time-consuming and costly, and, considering the large number of assays conducted on the fresh and stored samples, the number of test products had to be restricted.

In the present study, an evaluation of the stability of vitamin A in margarine was carried out using physico-chemical assay methods of demonstrated reliability *(3, 4, 5, 6)*. With the non-biological test procedures, a large number of samples could be and were assayed. Only illustrative data are herewith reported; these blanket operations are over a 1½-year period of margarine production in 3 plants and are sufficient in number to permit broad generalizations. Interest is centered in the present study on the retention of vitamin A in margarine during the shelf life of the product under practical conditions of storage. Attention is given to the retention of both forms of vitamin A, the preformed vitamin and provitamin-carotene.

EXPERIMENTAL

As a routine control on the vitamin A fortification of margarine manufactured on a commercial scale, tests are conducted daily on the product by non-biological assay procedures of demonstrated reliability *(4, 5)*. These tests involve assays of the fortified oils, covering a full day's production of white and yellow margarine, and of samples of each of the final products. In addition, 2 samples of each type of product are stored each month at 45°F. for a period of 3 months and the assays are then repeated. These holding tests blanket the shelf life of the product and give a reliable practical picture of the stability of vitamin A in margarine. For these tests the corresponding unfortified margarine oils are similarly stored. These are used to correct the spectrophotometric measurements for the presence of irrelevant light absorbing materials.

In Table 1 are listed the results obtained in testing 36 different samples of uncolored margarine before and after storage at 45°F. These samples cover the production

TABLE 1

Retention of vitamin A in uncolored margarine during storage of the product over a period blanketing shelf life

Production*		Vitamin A content		Loss of vitamin A during storage
		Fresh product	After 3 months at 45°F.	
Year	Month	USP units per lb. of margarine		
1951	April	16,700	16,700	0
		17,200	17,000	200
	March	17,200	17,000	200
		16,600	16,600	0
	February	17,200	16,600	600
		17,200	17,500	0
	January	18,000	18,000	0
		17,500	17,500	0
1950	December	17,200	17,200	0
		17,000	17,200	0
	November	17,000	16,600	400
		17,000	16,700	300
	October	17,400	17,400	0
		17,400	17,200	200
	September	16,800	16,800	0
		17,200	16,200	1,000
	August	16,800	16,200	600
		16,700	16,400	300
	July	17,400	16,600	800
		16,600	16,600	0
	June	16,800	16,600	200
		16,400	16,600	0
	May	16,200	16,200	0
		15,800	15,700	100
	April	15,000	15,700	0
		16,400	16,400	0
	March	16,700	16,000	700
		16,700	15,500	1,200
	February	17,000	16,400	600
		16,700	15,800	900
	January	16,000	15,300	700
		17,200	16,700	500
1949	December	16,700	16,000	700
		17,500	17,200	300
	November	17,300	17,000	300
		16,700	16,400	300
Average				[illegible]
Standard deviation				±340

*Non-selected illustrative data accumulated in controlling the vitamin A fortification of uncolored margarine produced in Plant B.

of one plant during a period of 18 months just prior to the writing of this report. Thirty-six samples of yellow margarine were also analyzed before and after storage. The results of analyses of the yellow margarine, listed in Table 2, permit an evaluation of the stability in margarine of not only preformed vitamin A but also of carotene (provitamin A).

From the data in both Tables 1 and 2 it may be seen that vitamin A as such or as the provitamin is very stable in margarine when properly stored (at 45°F.) for a period blanketing the shelf life of the product. The loss of 2 to 3% of vitamin A or carotene is negligible and is more than compensated for by the overage in fortification.

TABLE 2

Retention of vitamin A and carotene in yellow margarine during storage of the product over a period blanketing shelf life

Production[b]		Vitamin A content				Loss of vitamin A during storage	
		Preformed vitamin A		Carotene			
		Fresh product	After 3 months at 45°F.	Fresh product	After 3 months at 45°F.	Preformed vitamin A	Carotene
Year	Month	USP units per lb. of margarine					
1951	April	11,200	11,200	6,600	6,500	0	100
		11,700	11,500	7,200	7,000	200	200
	March	10,500	10,400	6,300	6,300	100	0
		10,800	10,800	7,400	7,200	0	200
	February	11,900	11,700	6,300	6,300	200	0
		11,000	10,800	6,400	6,000	200	400
	January	11,200	11,200	6,600	6,500	0	100
		11,000	10,600	6,700	6,500	400	200
1950	December	11,700	11,500	6,800	6,500	200	300
		11,300	11,700	6,700	6,500	0	200
	November	11,000	10,800	6,900	6,300	200	600
		11,700	11,000	6,600	6,500	700	100
	October	11,700	11,300	6,800	6,700	400	100
		11,700	11,300	6,500	6,500	400	0
	September	11,200	10,600	6,600	6,200	600	400
		11,500	11,200	6,500	6,300	300	200
	August	11,200	10,600	7,000	6,800	600	200
		11,900	11,000	7,200	7,300	900	0
	July	10,600	10,800	6,500	6,000	0	500
		10,600	11,000	6,600	6,300	0	300
	June	11,700	12,600	6,600	6,600	0	0
		10,600	11,200	6,700	6,600	0	100
	May	10,300	10,200	6,900	6,800	100	100
		10,300	10,300	6,900	6,700	0	200
	April	11,300	11,000	6,800	6,600	300	200
		10,600	10,500	6,900	6,600	100	300
	March	10,200	10,800	6,900	6,400	0	500
		10,600	11,000	6,800	6,600	0	200
	February	11,900	12,200	7,300	6,900	0	400
		11,500	11,700	6,900	6,800	0	100
	January	10,500	10,300	6,800	6,600	200	200
		11,200	10,500	5,900	6,100	700	0
1949	December	10,200	9,900	6,500	6,400	300	100
		10,600	10,300	6,600	6,400	300	200
	November	11,200	11,300	5,500	5,500	200	0
		10,500	10,200	6,800	6,600	300	200
Average						219	191
Standard deviation						±240	±157

[b] Non-selected illustrative data accumulated in controlling the vitamin A fortification of yellow margarine produced in Plant B.

That the results obtained with the production from one plant are typical of what is produced as a whole, is shown by the data presented in Table 3 covering the production from 3 plants located in 3 different parts of the country.

Unfortunately margarine is not always stored in the refrigerator. In some retail outlets the product may be held at room temperature for a number of weeks prior to sale. In Table 4 are presented the results of a study on the stability of vitamin A and carotene in margarines held under abusive storage conditions, i.e., one month at 75°F.

TABLE 3

Summation of stability studies of vitamin A and carotene in margarines produced in Different plants over a period of 18 months

Vitamin A stability evaluation	Plant A	Plant B	Plant C
Uncolored margarine			
No. of samples tested	36	36	36
Average vitamin A values, USP units/lb.			
Fresh samples	17,200	16,900	17,250
Same after 3 months at 45°F.	17,200	16,600	17,000
Loss of vitamin A	45	308	253
Standard deviation	±239	±340	±307
Average retention of vitamin A, %	99.7	98.2	98.5
Yellow margarine			
No. of samples tested	36	36	36
Average values for preformed vitamin A, USP units/lb.			
Fresh samples	11,900	11,100	11,100
Same after 3 months at 45°F.	12,100	11,000	11,100
Loss of vitamin A	36	219	167
Standard deviation	±82	±240	±270
Average retention of preformed vitamin A, %	99.7	98.0	98.5
Average values for carotene, as USP units vitamin A/lb.			
Fresh samples	5,700	6,700	6,750
Same after 3 months at 45°F.	5,500	6,500	6,500
Loss of carotene	228	191	250
Standard deviation	±204	±157	±178
Average retention of carotene, %	96.0	97.1	96.3

It was not considered realistic to store the products at higher temperatures, since oil staining of the carton makes the product unsalable. As a further check on the reliability of the vitamin A values found, the tests included assays by 2 unrelated analytical procedures. The test methods are briefly described in the footnotes to Table 4; for a full description of the methods the reader is referred to the original publications *(4, 5)*. The test samples were 12 in number and consisted of 2 uncolored and 2 yellow margarines drawn from the regular production of each of the 3 plants.

The earlier publications *(4, 5)* had clearly demonstrated that the spectrophotometric method employed for estimating vitamin A content gave reliable figures while those based on the chromogenic reaction between vitamin A and antimony trichloride were somewhat in error. The colorimetric values for preformed vitamin A were about 4% too high despite fortification with quality vitamin A concentrates *(6)*. It was tentatively suggested *(4)* that some conversion of vitamin A to the unsaturated hydrocarbon, anhydro vitamin A, occurs in the manufacture of margarine with fully cultured milk. This vitamin A derivative is practically free of biological activity, has a strikingly different light absorption curve than that of vitamin A, but reacts with antimony trichloride to almost the same degree as the vitamin itself *(7)*. Hence, the formation of such a compound in margarine manufacture could readily explain lower spectrophotometric values relative to those obtained colorimetrically, despite equivalence in assays of the fortifying concentrates. The margarines used in the present study, the results of which have been summarized in Table 4, were less acid in reaction than those covered in previous reports *(4, 5)*, having been made with milk cultured to only ⅓ of the practical maximum. This may explain the better agreement in the results currently obtained by the 2 assay methods, particularly in tests of the uncolored margarines. However, given sufficient time (one month at 75°F.) a significant discrepancy between colorimetric and spectrophotometric estimates develops. Thus, the spectrophotometric values indicate a vitamin A loss of about 8%, while the colorimetric values show a destruction of only about 3%. These observations support further the hypothesis that the conversion of vitamin A to anhydro vitamin in margarine, not detectable by the colorimetric ($SbCl_3$) test, is of practical significance in an under-

TABLE 4

Stability of vitamin A and carotene in margarines produced in different plants, held under abusive storage conditions, and assayed by two different test methods

Vitamin A stability evaluation	Plant A		Plant B		Plant C		Average values
	1st sample	2nd sample	1st sample	2nd sample	1st sample	2nd sample	
Uncolored margarine—spectrophotometric test [c]							
Vitamin A values, USP units/lb.							
Fresh sample	17,200	17,500	18,000	17,700	18,000	17,800	17,700
Same after 1 month at 75°F.	15,800	16,700	17,300	15,500	16,400	15,800	16,250
Loss of vitamin A	1,400	800	700	2,200	1,600	2,000	1,450
Retention of vitamin A, %	91.8	95.4	96.1	87.6	91.1	88.8	91.8
Uncolored margarine—colorimetric ($SbCl_3$) test [d]							
Vitamin A values, USP units/lb.							
Fresh sample	18,300	17,400	17,200	17,800	17,900	17,200	17,600
Same after 1 month at 75°F.	17,200	16,700	17,400	17,300	16,700	16,700	17,000
Loss of vitamin A	1,100	700	0	500	1,200	500	650
Retention of vitamin A, %	94.0	96.0	100.0	97.2	93.3	97.1	96.3
Yellow margarine—spectrophotometric test [e]							
Values for preformed vitamin A, USP units/lb.							
Fresh sample	11,900	11,900	10,600	10,500	10,600	10,300	11,000
Same after 1 month at 75°F.	10,500	10,800	10,600	9,900	9,300	9,100	10,000
Loss of vitamin A	1,400	1,100	0	600	1,300	1,200	950
Retention of preformed vitamin A, %	88.2	90.7	100.0	94.3	87.7	88.4	91.6
Values for carotene, spectrophotometric test, USP units vitamin A/lb. [e]							
Fresh sample	5,800	5,800	5,900	6,600	6,000	6,300	6,100
Same after 1 month at 75°F.	5,300	5,200	5,500	6,400	5,700	6,000	5,800
Loss of carotene	500	600	400	200	300	300	400
Retention of carotene, %	91.4	89.7	93.2	97.0	95.0	95.2	93.6
Yellow margarine—colorimetric ($SbCl_3$) test [f]							
Values for preformed vitamin A, USP units/lb.							
Fresh sample	12,500	12,600	11,500	11,500	11,000	11,000	11,700
Same after 1 month at 75°F.	12,000	11,700	11,400	11,700	11,000	11,300	11,500
Loss of vitamin A	500	900	100	0	0	0	250
Retention of preformed vitamin A, %	96.0	92.8	99.1	100.0	100.0	100.0	98.0
Values for carotene, spectrophotometric test, USP units vitamin A/lb. [g]							
Fresh sample	5,400	5,300	5,400	6,100	5,900	6,000	5,800
Same after 1 month at 75°F.	5,000	4,800	5,100	5,900	5,500	5,600	5,300
Loss of carotene	400	500	300	200	400	400	400
Retention of carotene, %	92.6	90.6	94.4	96.7	93.2	93.3	93.5

[c] Assays involving light absorption readings at 328 mμ of the whole oil separated from the margarine and dissolved in cyclohexane, the spectrophotometer having been set at 100% transmission with the corresponding unfortified oil of similar history *(4)*.

[d] Assays involving the reaction in chloroform solvent of antimony trichloride with the vitamin A in the unsaponifiable extract of the oil separated from the margarine, the resulting blue color being translated into vitamin A unitage by referring to a vitamin A calibration curve *(4)*.

[e] As the first footnote above, but with light absorption readings taken at 455 mμ *(5)*.

[f] As the second footnote above, but correction made for the chromogen developed as a result of the reaction between antimony trichloride and carotene *(5)*.

[g] Assays involving light absorption readings at 455 mμ of the unsaponifiable extract of the oil separated from margarine, with a fixed correction for irrelevant light absorption *(5)*.

standing of the fate of vitamin A in margarine during manufacture and storage. The colorimetric estimates of the vitamin A content, particularly of stored margarine, are regarded to be erroneously high but of confirmatory value when spectrophotometric figures are available. In the absence of the latter figures the colorimetric estimates should be regarded as the maximal values for vitamin A content.[b]

Provitamin A, carotene, exhibits a stability in margarine comparable to that noted with preformed vitamin A. Under abusive storage conditions (one month at 75°F.) about 7% of the carotene is lost in comparison to the 8% noted in the case of preformed vitamin A. [The latter figure discounts the obviously high retention values obtained colorimetrically ($SbCl_3$ test) for preformed vitamin A.]

The margarine manufacturer must in fortifying his product add a sufficient overage of vitamin A to compensate for processing and storage losses, recognizing that the product may be held at times under abusive storage conditions. From the results of the present study, it can be concluded that margarine must be fortified with quality vitamin A and carotene concentrates *(5, 6)* to a level of at least 17,000 USP units of vitamin A per pound, and preferably to the 18,000 USP unit level, to provide sufficient vitamin A overage to cover processing and storage losses and the occasional aberrant values obtained in assays of the product before and after storage. Our own experiences have shown that this policy will furnish margarines that never fall below the minimal 15,000 USP unit level in the label declaration throughout the shelf life of the product.

The negligible loss of 2 to 3% of vitamin A or carotene when margarines are held for 3 months at 45°F. compares favorably with that noted for the better butters, those prepared from sweet cream *(1)*. When neutralized sour cream is employed for buttermaking, losses of about 10% of vitamin A are noted following storage of the butters for one to 2 months at 40°F. *(1)*. Since "more butter is made from sour cream than is made from sweet cream" *(1)*, it may be concluded that vitamin A is in general more stable in margarine than in butter.

SUMMARY

Vitamin A as such or as the provitamin, carotene, is stable in margarine. Fully 97 to 98% of the vitamin is retained during the shelf life of the product when held under refrigeration (45°F.) storage. When margarine is abused during storage, held at room temperature (75°F.) for a period of one month at which point oil-staining of the carton renders the product unsalable, vitamin A retention still averages well above 90%. Evidence was presented that in margarine, prepared with cultured milk, conversion of vitamin A to the biologically-inactive anhydro vitamin A can be responsible for the somewhat erroneously high vitamin A estimate by the colorimetric ($SbCl_3$) test procedure. Comparison of the results reported in the present investigation with those published by others has justified the conclusion that vitamin A is in general more stable in margarine than in butter.

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THERMAL INACTIVATION OF PECTOLYTIC ENZYMES USED IN THE CLARIFICATION OF APPLE JUICE[a]

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During an investigation on the nature and causes of sediment formation in clarified apple juice the thermal inactivation of enzymes commonly used in the clarification treatment was studied. Thermal inactivation data for the pectin-esterase (PE) and polygalacturonase (PG) fractions of a commercial enzyme preparation of fungal origin (Pectinol A)[b] were obtained. Some investigations have suggested that sediment formation in bottled or canned clarified apple juice during storage might be caused by the action of pectolytic enzymes that survived the pasteurization treatment.

Pederson and Tressler *(13)* suggested that sediment formation in processed apple juice might be caused by the continued action of the clarifying enzymes if the enzymes were not inactivated during the pasteurization of the juice. Cruess, Aref, and Irish *(2)* and Joslyn and March *(8)* pointed out that when pectin-splitting enzymes are used for the clarification of apple juice, too much of the enzyme preparation should not be added since it may be responsible for some precipitation. After clarification by enzymes, the juice must be heated to 150°F.(65.6°C.) to stop any further action of the enzymes. Marshall *(12)* indicated that flash-heating apple juice to 190°F.(87.8°C.) inactivated Pectinol A when it was used as a clarification agent.

Tressler, Joslyn, and Marsh *(15)* reported that if Pectinol-clarified cider is to be kept for any length of time after bottling, it should be heated at 170°F.(76.7°C.) for 30 minutes to inactivate the enzyme remaining in the juice, or otherwise the juice may cloud again because of continued enzyme action. It was also reported that the enzymes of Pectinol require 10 minutes at 160°F.(71.1°C.) for complete inactivation. Kertesz *(11)* and Willaman and Kertesz *(16)* found that the activity of a pectin-destroying enzyme produced by *Penicillium glaucum* Link was inactivated by heating for 30 minutes at 131°F.(55°C.). Relationships of pectin and other factors concerned with clouding and sedimentation in apple juice have been reviewed by Forgacs, Ruth, and Tanner *(4)*. Carpenter and Walsh *(1)* reported that apple juice treated with an enzyme preparation, Clarase, must be heated to 150°F.(65.6°C.) to inactivate the enzyme. Joslyn, Mist, and Lambert *(9)* have presented evidence for the natural occurrence of polygalacturonase and pectinesterase enzymes in apple juice. Pollard and Kieser *(14)* reported that the pectin demethoxylase content of different varieties of apples varied considerably. A purified preparation of this enzyme was more readily destroyed by heat than the enzyme in raw apple juice.

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[a]Contribution No. 883, Massachusetts Agricultural Experiment Station.
[b]Supplied by Rohm and Haas Company, Philadelphia, Pa.

EXPERIMENTAL METHODS

Apple juice substrate. Thermal destruction tests on the pectolytic enzymes were made on apple juice. The juice was prepared with a laboratory type cider mill and press. The juice was strained through cheesecloth and was then heated for 20 minutes at 210°F.(99°C.) to inactivate any naturally occurring pectolytic enzymes. The tests on the thermal destruction of PE were carried out with juice from McIntosh apples that had a pH value of 3.58 and a soluble solids content of 11.9%. A blend of Baldwin and McIntosh apple juices (with a pH value of 3.82 and a soluble solids content of 10.5%) was used for the tests on PG (polygalacturonase).

Thermal destruction tests. The technique followed in the thermal destruction tests was essentially that described by Kaplan, Esselen, and Fellers *(10)*. Portions of the apple juice plus the enzyme preparation were placed in rimless Pyrex test tubes 125 mm. long and 15 mm. outside diameter or in Pyrex "thermal death time tubes" 150 mm. long and 9 mm. outside diameter. The tubes were heated in a constant temperature oil bath for the times and temperatures indicated and were then cooled in ice water. Heating lag correction factors for each size of tube were determined as described by Kaplan, Esselen, and Fellers *(10)*. Lag correction factors of 6.52 and 2.3 minutes, respectively, were applied to the heating times of the test tubes and thermal death time tubes.

Pectin esterase (PE). Apple juice was treated with Pectinol A in the proportion of 10 per cent enzyme preparation to 90% of juice by weight, for several hours at 77°F.(25°C.). The juice was then filtered through No. 50 Whatman filter paper with a Büchner suction funnel. Ten-ml. portions of the clarified juice were placed in the Pyrex test tubes, and the tubes were heated for varying lengths of time at different temperatures as indicated in Table 1. During heating, the tubes were positioned in the oil bath so that the surface level of apple juice was 35 mm. below the surface of the surrounding oil. The PE activity of the various lots of heated apple juice was determined by the method of Fish and Dustman *(3)*.

Pectin polygalacturonase (PG). Apple juice was clarified with a sugar-free pectinol A, in the proportion of 0.8% of the weight of the juice; during a 4-hour reaction period at 77°F.(25°C.). A sugar-free enzyme preparation was prepared by removing the dextrose from commercial Pectinol A, as described by Fish and Dustman *(3)*. Pyrex test tubes containing 10-ml. portions of the filtered juice were heated for varying lengths of time at different temperatures as indicated in Table 2. In the tests at 170°F.

TABLE 1

Thermal inactivation of pectin esterase (PE) of 10% Pectinol A in apple juice

Temperature	Time	
	PE active	PE inactive
°F.	*min.*	*min.*
135	155	160
140	52	54
145	18	19
150	7	8
155	0	4.75

TABLE 2

Thermal inactivation of pectin polygalacturonase (PG) of 0.8% sugar-free Pectinol A in apple juice

Temperature	Time	
	PG active	PG inactive
°F.	*min.*	*min.*
150	250	260
155	90	92
160	21	22
165	9	10
170	4.5	5.0

(76.7°C.), thermal death time tubes containing 2-ml. portions of juice were used, rather than the larger test tubes with their larger heating lag correction because of the short heating time required for destruction. The contents of 5 tubes that had received the same heat treatment were combined for making the PG determinations. The PG activity of the samples of heated apple juice was determined by adding the juice to a pectic acid substrate and measuring its increase in reducing value over a 24-hour incubation period at 77°F.(25°C.) by the hypoiodite method of Goebel *(5)*.

RESULTS

The data obtained are listed in Tables 1 and 2. Semilogarithmic thermal inactivation curves for PE and PG, based on these data, are presented in Figure 1. The curves have been characterized in terms of their F_{150} and z values:

F_{150} = time required to inactivate enzyme at 150°F. in the specific medium.

z = slope of curve = number of degrees F. required for the curve to traverse one log cycle.

The PE enzyme had an F_{150} value of 7.2 minutes and z value of 11.3°, whereas the PG enzyme had an F_{150} value of 255 minutes and a z value of 11.5°. Thus, the PG was considerably more heat-resistant than the PE, and pasteurization treatments sufficient to destroy the PE would not necessarily inactivate the PG activity.

In the above tests, the concentration of PE was much higher than that normally added to apple juice for clarification. This high concentration was used in order to obtain a sufficient amount of enzyme in the apple juice so that it could be readily measured.

Joslyn, Mist, and Lambert *(9)* have recently reported studies on the clarification of apple juice by fungal pectic enzyme preparations. When selected enzymes were applied in the clarification of apple juice, no relation between PG or PE activity and rate or type of clarification was noted. Jansen and MacDonnell *(6)* and Jansen, MacDonnell, and Jang *(7)* had previously observed that the hydrolysis of pectin by a mixture of PG and PE was dependent on the rate of hydrolysis of methyl bonds by the PE.

Until the mechanism of clarification of fruit juices by the use of fungal enzymes is better understood, the desirability of inactivating all the enzymes concerned in the subsequent pasteurization treatment is indicated. The present data indicate that the tests for adequacy of pasteurization, from this standpoint, should be based on the inactivation of the PG present in the clarified juice on the basis that it is much more heat-resistant than the PE. Pasteurization at 180°F.(82°C.) for 0.6 and 1.0 minute, or its equivalent at other temperatures is indicated for the enzyme preparation and apple juices used in the present study. The effect of variations in the composition of different apple juices and of different enzyme preparations is not known.

SUMMARY

The thermal destruction characteristics of pectin esterase (PE) and pectin polygalacturonase (PG), from a commercial pectic enzyme preparation, in apple juice were determined. The PE was much more readily destroyed by heat than was the PG.

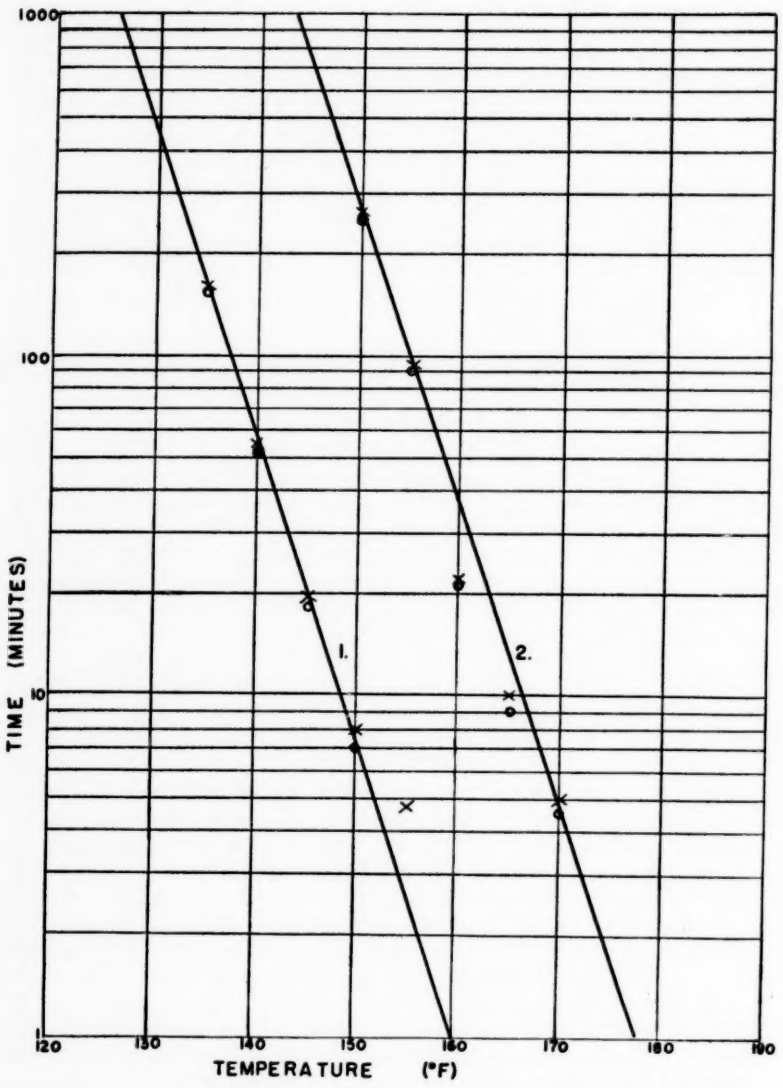

Figure 1. Thermal destruction time curves for pectin esterase and pectin polygalacturonase in apple juice.

1. Pectin esterase. 2. Pectin polygalacturonase.

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A STUDY OF PROTEOSE PEPTONE ACID AGAR AS A PLATING MEDIUM FOR THE ROUTINE ENUMERATION OF *BACILLUS THERMOACIDURANS* (BERRY) IN TOMATO JUICE*

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Proteose peptone acid agar was devised by Stern, Hegarty, and Williams *(7)* for the detection and cultivation of *Bacillus thermoacidurans* (Berry). They noted that, because contaminating types of bacteria were rarely found, direct plating of tomato juice with this medium was possible. During their studies they consistently observed that unless *B. thermoacidurans* (Berry) was present in the tomato juice samples, plates poured with proteose peptone acid agar appeared to be sterile. The use of this medium, therefore, gave both a qualitative and quantitative picture of a spoilage outbreak.

Cameron and Bohrer *(4)* advocated the use of proteose peptone acid agar for the detection of *B. thermoacidurans* (Berry) in their spoilage control procedures. They claimed that this medium promoted the growth of the "flat-sour" type of bacteria and, when used together with a heat treatment of the sample being analyzed, inhibited the growth of bacterial types not capable of producing spoilage of tomato juice. They found that the plate counts observed in this medium represented the number of *B. thermoacidurans* (Berry) and other acid tolerant types of bacteria present in tomato juice, and gave an index of the spoilage potential of the sample being analyzed. They did not, however, suggest the necessity of distinguishing between *B. thermoacidurans* (Berry) and other acid tolerant types. They concluded that a plate count of 10 colonies per ml. on proteose peptone acid agar represented a maximum limit of normal contamination and plate counts exceeding this limit indicated the possibility of some degree of spoilage. The spoilage potential of a sample became greater with increasing plate counts on this medium. The limit of 10 colonies per ml. was later changed *(5)*. The number of plates, each inoculated with one ml. of tomato juice, poured with proteose peptone acid agar, and incubated at 55°C.(131°F.) was increased from 3 to 6, and from experience they found that any colonies per 6 ml. of tomato juice was indicative of possible spoilage and should be investigated.

During the winter of 1951-1952, several hundred cans of tomato juice were examined in this laboratory following the procedures suggested by Cameron and Bohrer *(4, 5)*. Colonies were picked from proteose peptone acid agar plates and transferred to slants of the same medium to collect cultures of *B. thermoacidurans* (Berry) for heat resistance studies. Upon examination of the large number of organisms isolated, relatively few cultures proved to be typical of this organism; the remainder were mor-

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phologically quite unlike cultures of *B. thermoacidurans* (Berry). No attempt was made at that time to study these organisms.

This investigation was undertaken during our 1952 survey of commercial tomato juice processing lines, to decide whether or not a further differentiation of the organisms growing on proteose peptone acid agar should be made when conducting a line survey. Our object was to determine more accurately the hazard of contamination of the plant equipment with *B. thermoacidurans* (Berry).

EXPERIMENTAL

The dehydrated medium, thermoacidurans agar,[b] of the same composition as the medium of Stern, Hegarty, and Williams *(7)* was used throughout this investigation. Dextrose tryptone agar[b] was used in the isolation of pure cultures by serial plating. Becker and Pederson *(1)* used a sugar deficient tryptone, glucose, yeast extract agar to obtain profuse spore production. In this investigation a spore medium containing the following constituents per 1,000 ml. was used: dextrose, 0.1 g.; dibasic potassium phosphate, 4.0 g.; proteose peptone, 5.0 g.; yeast extract, 5.0 g.; agar, 20 g. The first 4 constituents were dissolved in 500 ml. of distilled water and adjusted to pH 5.0 with HCl. The agar was dissolved in a separate flask in the remaining 500 ml. of water. Following the sterilization of both solutions at 15 pounds' pressure for 15 minutes, the agar was aseptically mixed with the other constituents. The medium was then aseptically tubed, and slanted in the usual manner.

All samples of tomato juice taken from sampling points along the processing lines of all plants being surveyed, except processed cans, were given a heat treatment of 7 minutes in flowing steam. One ml. of each sample was then pipetted into each of three sterile petri plates. The plates were poured with thermoacidurans agar and incubated for 48 hours at 55°C.(131°F.). At the end of this incubation time, colonies growing in these agar plates were counted by means of a Quebec colony counter and the numbers recorded. In only a few instances did the bacterial numbers exceed 2 or 3 per ml. One or 2 colonies were picked from the plates of thermoacidurans agar and transferred to slants of the same medium which were incubated at 55°C.(131°F.) for 16-20 hours. Films were then made from these cultures on glass slides, stained by the Gram method, and examined under the microscope. Cultures which appeared to be typical of *B. thermoacidurans* and doubtful cultures were placed in a separate rack. Slants of the filamentous type organisms were placed in a second rack and slants of the miscellaneous rod type organisms were set in a third rack. All cultures were transferred to slants of the spore medium and stored for future attention.

At the end of the packing season these organisms were transferred to fresh slants of thermoacidurans agar and incubated 16-20 hours at 55°C.(131°F.). Serial dilutions were then plated with dextrose tryptone agar[b] and the plates were incubated at 55°C.(131°F.) for 24 hours. Well isolated colonies were picked from suitable plates, transferred to thermoacidurans agar slants, and incubated for 16-20 hours at 55°C. (131°F.). A heavy inoculum of each resulting culture was transferred by means of a sterile inoculating loop to each of 2 tubes of tomato juice which had been adjusted to a pH of 4.5 and autoclaved for 15 minutes at 15 pounds' pressure. One set of tubes was incubated at 55°C.(131°F.), and the other set at 37°(98°F.) for 14 days. At the same time slants of the spore medium were inoculated with these organisms and incubated at 55°C.(131°F.) for 7 days, or longer when necessary. Films were made from each culture on glass slides, stained with Schaeffer and Fulton spore stain, and examined microscopically. The pH of the tubed tomato juice was determined at the end of the 14-day incubation time. "Flat-sour" spoilage was recognized by a definite increase in the hydrogen ion concentration of the tubed tomato juice.

A heavy inoculum of a 16- to 20-hour culture of each organism which caused a decrease in the hydrogen ion concentration of the tubed tomato juice was transferred by means of a sterile inoculating loop to tubes of sterilized tomato juice which had

[b]Manufactured by Difco Laboratories.

been adjusted to pH 4.35, pH 4.25, and pH 4.20, respectively. These tubes were incubated for 14 days at the incubation temperature at which the original pH change occurred. The pH was again determined at the end of the 14-day incubation time.

A Beckman glass electrode pH meter was used for all pH measurements.

RESULTS AND DISCUSSION

Of the 41 original isolations of the filamentous type only 27 were recovered upon retransfer to fresh thermoacidurans agar slants. The morphology of all these cultures resembled that of Actinomycetes (Plate I: Figs. 5, 6). No growth and no pH change occurred in the tubes of tomato juice incubated at either 37°C.(98°F.) or at 55°C.(131°F.). Twelve of the recovered cultures showed definite spore formation. The filamentous type organisms were found in samples taken from all parts of the tomato juice processing line except the cooling water.

Of the 40 cultures regarded as miscellaneous Gram positive and Gram negative rods, 24 caused no significant change in the hydrogen ion concentration of the tubed tomato juice. Sixteen cultures caused a decrease in hydrogen ion concentration resulting in final pH values of from 4.51 to 4.91. Seven of these organisms failed to grow in dextrose tryptone agar, and thermoacidurans agar had to be used for plating the serial dilutions. All but 9 cultures showed spore production.

Both the microscopical examination and the results of the incubation tests indicated that none of the filamentous type organisms and none of the miscellaneous rod type organisms isolated from thermoacidurans agar were *B. thermoacidurans* (Berry).

Of the 27 cultures regarded as *B. thermoacidurans* (Berry), 22 caused definite "flat-sour" spoilage, 3 caused a limited acid production, one caused no pH change, and one caused a decrease in the hydrogen ion concentration of the tubed tomato juice. All but 2 cultures showed spore production. The 2 cultures which did not increase the hydrogen ion concentration of the tubed tomato juice were not considered to be *B. thermoacidurans* (Berry). The remaining cultures showed a varying ability to produce acid at 55°C.(131°F.) and the greatest increase in hydrogen ion concentration always occurred at 37°C.(98°F.) thus bearing out the work of Berry *(2, 3)*, Becker and Pederson *(1)*, and Cameron and Bohrer *(4)*.

The cultures which caused a decrease in the hydrogen ion concentration of tomato juice adjusted to pH 4.50 did not do so in tomato juice adjusted to pH 4.32, pH 4.25, or pH 4.20.

Microorganisms growing in thermoacidurans agar can roughly be divided into 3 groups on the basis of their morphological characteristics when examined under the microscope after 16-20 hours' incubation on thermoacidurans agar slants. *B. thermoacidurans* (Berry) can be recognized by its relatively long cells occurring singly, in pairs, or in short chains, which generally stain partly Gram positive and partly Gram negative (Plate I: Figs. 1, 2). This staining characteristic has been found to be consistent in most cultures of this age. The younger cells stain Gram positive and the older cells stain Gram negative. There are exceptions to this; occasionally a whole smear will stain Gram positive or Gram negative but even without this distinguishing characteristic the morphology of 16- to 20-hour cultures is such that they cannot be readily

confused with cultures of the same age of the other 2 types, which are either fine tangled filaments (Plate I: Figs. 5, 6) or short rods, the majority of which stain Gram negative (Plate I: Figs. 3, 4).

Differentiation of these organisms is of importance when reporting the results of a line survey because the filamentous type and the miscellaneous rod type organisms are not themselves the cause of "flat-sour"

Plate I. Organisms isolated from thermoacidurans agar (Difco).

Figure 1. Culture No. 400.

Figure 2. Culture No. 591.

Figures 1, 2. Photomicrographs of B. thermoacidurans type organisms isolated from thermoacidurans agar (Difco). × 1400.

Figure 3. Culture No. 546.

Figure 4. Culture No. 728B.

Figures 3, 4. Photomicrographs of miscellaneous rod type organisms isolated from thermoacidurans agar (Difco). × 1400.

Figure 5. Culture No. 772.

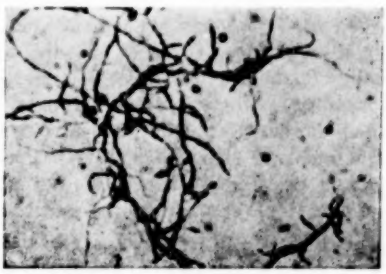

Figure 6. Culture No. 710B.

Figures 5, 6. Photomicrographs of filamentous type organisms isolated from thermoacidurans agar (Difco). × 1400.

spoilage of tomato juice. When these organisms are recovered from processing line samples alone in such low numbers as one or two per 3 plates of thermoacidurans agar[b] their importance in regard to "flat-sour" spoilage of tomato juice is limited. However, when the numbers of these organisms are high they do indicate a condition of improper sanitation which presupposes the presence of *B. thermoacidurans* even though it is not recovered from the incubated agar plates. Such a condition should still be considered dangerous.

The pH of tomato juice appeared to be a limiting factor in the growth of those organisms which caused a decrease in the hydrogen ion concentration of tomato juice adjusted to pH 4.5. None of these organisms caused an alkaline reaction in tomato juice adjusted to pH 4.20, pH 4.25, or pH 4.32. Some of these organisms, however, may be responsible for off-flavors in tomato juice having a normally high pH. This is particularly true of those organisms which caused the alkaline reaction at 37°C.

None of the filamentous type organisms inoculated into sterile tomato juice at pH 4.5 caused any significant change in pH and so had no significance either in regard to "flat-sour" spoilage or to spoilage of an alkaline nature. According to Waksman *(8)* the heat resistance of Actinomycetes spores is relatively low, 60°-65°C.(140°-150°F.) for 10 to 15 minutes, so that their presence in filler bowls and processed cans may be an indication of underprocessing. However, the thermal resistance of these organisms has not yet been determined. Some of these cultures did not produce spores when grown on the thermoacidurans agar slants. That they are spore formers, however, is quite probable, but this medium and the conditions of growth may not have been conducive to spore formation by these organisms.

Whether or not the filamentous types grow to any extent in the plant equipment is not known. No evidence of a rapid growth of these organisms was noted during this line survey. Their origin is probably the soil and they may pass through the processing line in the spore stage. They may, however, be present in the dust of the air, over beams in the plant, and in the empty cans.

SUMMARY

Thermoacidurans agar[b] is not specific for *B. thermoacidurans* (Berry), when used to conduct bacteriological surveys of commercial tomato juice processing lines. Colonies growing on this agar should not be recorded as being *B. thermoacidurans* (Berry) without being first transferred to thermoacidurans agar slants, incubated 16-20 hours at 55°C.(131°F.), and then examined under the microscope. While the recovery of only filamentous and miscellaneous rod type organisms in such small numbers as one or two per 3 plates is of limited importance, their recovery in higher numbers must still be considered to indicate a hazardous condition requiring further investigation.

Acknowledgment

Grateful acknowledgment is hereby made to H. Maeste, the author's assistant, for his valuable help in conducting this investigation.

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THE AMINO ACIDS IN VARIOUS TISSUES OF CITRUS FRUITS AND IN ORANGE PROTEIN[a]

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Nelson *et al. (15)* reported the presence of aspartic acid, choline, and small amounts of histidine in orange juice in addition to asparagine, glutamine, and stachydrin found previously by earlier investigators. Jansen and Jang *(6)* and Miller and Rockland *(14)* isolated cysteine and glutathione from orange juice. More recently the distribution of free amino acids in citrus juices was studied by filter-paper chromatography by Rockland *et al. (17)* and Underwood and Rockland *(23)*. The latter reported the occurrence of alanine, serine, glutamic acid, proline, and γ-amino butyric acid in addition to asparagine, aspartic acid, glutamine, arginine, and histidine, which have been reported previously. Underwood and Rockland *(23)* were interested primarily in a comparative investigation of the amino acids present in filtered juices of several varieties of citrus fruits.

Our investigations which were started in 1949, as a continuation of previously reported work on other fruits, Joslyn and Stepka *(8)*, were carried out for the purpose of (a) determining the distribution of free amino acids in the various tissues of several citrus fruits; (b) for the qualitative determination of changes in the amino acid constituents of juices that occur during the "browning reaction," and (c) for the analysis of the amino acid composition of the proteins isolated from the chromatophores and from the peel of the Valencia orange. These investigations, reported in detail in a thesis by P. M. Townsley *(22)*, are presented in summary in the present paper.

EXPERIMENTAL PROCEDURE

Preparation of fruit samples. Late Valencia oranges, early green Washington Navel oranges, late Marsh grapefruit, and tree matured Eureka lemons were picked early in October[b] and dissected into the following tissue preparations: albedo, flavedo, locular walls, juice vesicles, vascular tissue, seeds, and navel. In addition reamed juice was strained through cheesecloth, and chromatophores—separated from it by centrifuging, resuspending in water, and recentrifuging twice—were prepared.

As the tissues were removed from the fruit, they were dropped into sufficient volume of 95% alcohol to give a final concentration of from 70% to 80% alcohol, assuming all the different tissues, including juice, to contain 90% water. The samples then were blended in a Waring blendor for 5 minutes and stored at 0°C.

Aliquots of the filtered reamed juices were analyzed for Brix degree, titratable acidity with results as shown in Table 1.

Total nitrogen, excluding nitrate nitrogen was determined by the micro-kjeldahl method on each alcoholic extract after the alcohol precipitable matter and insoluble tissue had settled out. The data obtained are shown in Table 2 (nitrogen content of aliquot applied to paper for optimum chromatography of Valencia oranges).

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[a] Presented in part at the Thirteenth Annual Meeting of the Northern California Section, Institute of Food Technologists, Albany, California, December 4, 1952.

[b] The fruit was obtained from the orchard of the Citrus Experiment Station through the courtesy of Dr. W. H. Sinclair.

TABLE 1

Composition of citrus fruits tested

Citrus species	Acidity[c]	Brix	Soluble solids as sucrose[d]	Brix/acid
Late Valencia oranges	0.964	13.4	12.9	13.9
Early green Washington Navel oranges	2.035	10.3	9.9	4.1
Late grapefruit	1.197	9.5	8.8	7.9
Tree matured Eureka lemons	5.306	.....	8.2	1.5
Market ripe Valencia oranges	1.049	11.8	11.0	11.2

[c] Expressed as grams anhydrous citric acid per 100 ml.
[d] From refractive index measurements corrected for citric acid content.

TABLE 2

Total alcohol extractable nitrogen (mg./100 ml. of extract)

Tissue	Late Valencia oranges	Early green Washington navel oranges	Late grapefruit	Tree matured Eureka lemons
Flavedo	14.48	14.08	15.87	8.85
Albedo	10.91	5.79	7.66	6.86
Carpellary membrane	7.20	6.23	2.22	2.63
Vesicles	22.36	12.86	15.16	9.01
Vascular tissue	14.28	.....	9.13	11.41
Seeds	32.93	.....	22.62	9.13
Reamed juice	19.36	11.63	13.65	8.33
Centrifuged juice	19.69	11.82	.....	.....
Chromatophores	23.29	14.05	.....	.....
Navel	.....	14.92	.....	.....

Juice obtained from ripe Valencia oranges purchased on the local market (November, 1949) by reaming and straining through cheesecloth was stored in air as such after addition of 0.3% sodium benzoate and also after centrifuging to remove chromatophores. Alcoholic extracts of these 2 lots were prepared immediately and at several stages of darkening as observed both visibly and from measurements of optical density in a Klett photometer.

Proteins. Peels from the market Valencias used above, cleaned of all adhering membranes, and discolored injured tissue, were ground in a meat grinder to a fine pulp. An equivalent volume of 95% alcohol containing 1% sodium hydroxide (0.23 N) was added and the mixture blended in a Waring blendor for 3 minutes. The resultant suspension was stored overnight at 0°C. It was then filtered through fine meshed linen cloth, an equal volume of distilled water added and the pH adjusted to 4.7 with glacial acetic acid. The flocculated precipitate was separated by filtering through a fluted No. 2 Whatman filter paper. The precipitate was washed 4 times with 95% alcohol, twice with absolute ether, and then dried and stored in a desiccator. The crude protein was a yellow-colored powder; 6.47 g. were obtained from 3,500 g. of peel.

The peel protein was purified by redissolving in 0.3% sodium hydroxide, filtering through a No. 1 Whatman filter paper, and reprecipitating at pH 4.7 with glacial acetic acid; this was repeated 4 times. Each time the precipitate was washed with distilled water until no longer acid to litmus. The precipitate at the fourth filtration and washing was washed with 95% alcohol and then with absolute ether. It was dried in air and stored in desiccator over calcium chloride. The yield was 2.02 g. of a bright yellow-colored precipitate with a loss during purification of 66%.

The protein from the 280 ml. of chromatophores (separated by centrifuging 1 liter of Valencia orange juice at 2,000 r.p.m. for 30 minutes, syphoning off the juice and resuspending the residue in cold distilled water and recentrifuging) was isolated by adding an equal volume of 95% alcohol with 1% sodium hydroxide, filtering through linen cloth, and then precipitating with acetic acid as above. The yield of purified light cream colored protein was 2.33 g.

Each protein was hydrolyzed for chromatographic analysis by placing 0.2 g. of protein and 5 ml. of 6 N H_2SO_4, or 5 ml. of 6 N $Ba(OH)_2$, respectively, into sealed tubes. Hydrolysis was carried out for 16 hours in an autoclave at 15 pounds' pressure (120°C.). The barium and sulfate ions were quantitatively removed by the addition of sulfuric acid and barium hydroxide, respectively. The resulting solutions were neutral to litmus paper.

Chromatographic analysis. The technique employed for chromatographic analysis was that used by Joslyn and Stepka *(8)*. The solvent used in the first direction was water saturated phenol and in the second direction, butanol-acetic acid-water, of the following composition: 5 volumes of water, 1 volume of glacial acetic acid, and 4 volumes of n-butanol. To aid in the identification of the spots, a third solvent, 2-4 lutidine-water in a 1:1 ratio was used on a duplicate set of 8 papers for the second direction.

A preliminary series of 8 papers were run two dimensionally, using the first 2 solvents. The purpose was to determine the most ideal concentrate for analysis to be applied to the papers; it was closely estimated from the total nitrogen content of the extracts and from the amino acid fraction of the total nitrogen. This was assumed from the literature to be approximately 60%, Sinclair *et al. (18)*. Because the total nitrogen content of the extracts was low and varied from tissue to tissue, it was necessary to concentrate the extracts to previously calculated volumes. In order to do this, 2-ml. aliquots of the alcoholic extracts (in some cases fractional amounts of this 2 ml., depending on the amount of extract available, as with vasecular tissue) were placed in glass vials of dimensions 1¼ by ½ inch, and subjected to a vacuum of 28.5 inches at 35°C. Evaporation to the desired concentration required approximately 2 to 3 hours. The sample was then applied in 0.1-ml. amounts to the paper. This amount gave an area equal to a circle one cm. in diameter. A similar technique was employed for concentration and for application of all the alcoholic extracts analyzed in this work.

The identification of the spots was accomplished by several methods: by the pattern of the spots found by Dent *(3)* to be constant and reliable in 3 solvents with known markers naturally occurring in the extracts and by the addition of known amounts of amino acids* to the extracts before concentration. The extract was then chromatographed so that the naturally occurring amino acids would give only a faint pattern and the added amino acids could be easily detected by the increased intensity of a spot. In all cases the intensity of the spots was compared to controls developed at the same time to which no amino acids had been added.

The use of the 2 hydrolyzing agents, sulfuric acid and barium hydroxide, assisted in clarifying the identity of certain amino acid spots. Serine, threonine, and arginine are destroyed by barium hydroxide but are not destroyed by sulfuric acid under the conditions of hydrolysis. This disappearance of an amino acid from one hydrolysate and not in the other paralleled chemical tests, and thereby the identity of a spot could be established.

Before the ninhydrin was sprayed on the paper, the air-dried, non-heated papers were examined under ultraviolet light to map out the fluorescent areas.

DISCUSSION

The free amino acids of citrus fruit tissues. In carrying out the tests for the occurrence of free amino acids in citrus fruits some 70 to 80 chromatograms were made. Typical of the data obtained is that showing the distribution of amino acids in various tissues of Valencia oranges, Table 3.

Quantitative and possibly qualitative differences occurred in the free amino acid content of the citrus fruits examined. These differences were observed also from tissue to tissue. The qualitative differences occurred mainly in the basic amino acid fraction, which includes lysine, arginine,

*The amino acids were added before concentration so that they would be subjected to the same treatment as the naturally occurring amino acids and would most likely be in the same chemical equilibrium form, important in the amino acid movement in the solvents. This is especially true for the basic amino acids like arginine and lysine.

TABLE 3

Distribution of amino acids in late valencia orange tissues [f] (butanol-acetic acid : phenol-water)

Albedo[g]	Flavedo[g]	Carpellary membrane[g]	Vesicles[g]	Juice[g]
Ae, Al	Ae, Sn	An, Ae, Sn	Ae, Ar, Sn	Ar, Ae, Sn
Ln	An	Al, Ab, Ge	An	An
Ab, Sn	Ge	Ar	Ab	Ab
Ge, Pr	Al, Gn, Ab	Gn, Ls, Pr, U	Ge	Ge
An, Gn, Pn	Pr	Pn	Al	Al
U, Vn[h]	Pn, U	Tn	Pr	Pr
U, Ar. U[i]	Tn	Vn[h]	Ls	Ls
	Vn,[h] U		Vn[h]	Vn[h]
			Tn, Pn, Gn	Tn, Pn, Gn, U

[f] Arranged in order of approximate intensity of coloration with ninhydrin.

[g] Aliquot used for best separation contained the following amounts of nitrogen in mg.: Albedo—0.043; Flavedo—0.043; Carpellary membrane—0.028; Vesicles—0.017; Juice—0.025; Chromatophores—0.018. The amino acids identified were: Alanine—Al, Arginine—Ar, Asparagine—An, Aspartic acid—Ac, γ-amino butyric acid—Ab, Glutamine—Gn, Glutamic acid—Ge, Leucine—Ln, Lysine—Ls, Methionine—Mn, Ornithine—On, Phenylalanine—Pn, Proline—Pr, Serine—Sn, Threonine—Tn, Valine—Vn, and several unknowns—U.

[h] Valine and possibly methionine.

[i] This unknown may be ornithine.

ornithine (tentatively), the amide glutamine, and unknowns in this area on the papers. Arginine was the most variable of the known amino acids found. The amino acids, tyrosine and a spot corresponding to α-amino butyric acid were present in some extracts and not in others at the concentrations of the extracts analyzed. Tyrosine was found in the seeds of the Valencia, the Navel, the grapefruit, and in the flavedo and albedo of the Navel orange. Alpha-amino butyric acid was found in most of the tissues of the Navel orange but not in the free amino acid fraction of the extract of the other fruits. All extracts contained aspartic acid, glutamic acid, serine, threonine, alanine, γ-amino butyric acid, valine, and possibly methionine. With the valine there was phenylalanine, and in most cases the presence of one or more unknown members of the leucine group, proline and asparagine. The latter two, because of their characteristic color and reliable R_f values, were used as natural markers for the suggestion as to the identity of other amino acids.

Extracts from the juices of all the citrus fruit had to be chromatographed at lower levels of concentration because of the tendency to form "tails" upon development with phenol. The formation of "tails" did not occur in the butanol-acetic acid-water direction.

Preliminary chromatographs of tryptophane have been unsuccessful in locating the position of this amino acid, although the spot corresponding to valine increased slightly in intensity whenever tryptophane was added.

An excellent separation of the fluorescent substances was noted by observation under ultra-violet light. No attempt was made to identify them. The fluorescent spots differ qualitatively and quantitatively from extract to extract. Higher concentrations usually are associated with the flavedo, albedo, and vascular tissues of the fruit.

Free amino acids in the citrus alcoholic extracts. The free amino acids found in the alcoholic extracts of the various tissues may include the

easily hydrolyzable amino acids from the more complex units. The tissues were placed in alcohol within a few minutes after separation from the fruit, leaving only a short period in which enzymic hydrolysis could occur. Investigations by Webster *(25, 26)* indicate that there are some changes taking place in alcohol storage, but his preparations were under very adverse conditions and he presented no data on storage of alcoholic preparations at 0°C. At this temperature it is not likely that changes occur rapidly enough to destroy or alter the general picture of the free amino acids present.

The total extractable nitrogen in the alcoholic extracts varied from tissue to tissue, between tissues of a fruit, and between the fruits. The absolute nitrogen value will vary throughout the year, Cameron *et al. (2)*.

Seeds of the Valencia orange and the grapefruit contain more extractable nitrogen than other tissue. This is not so with the lemons, however, the nitrogen content of the vascular tissue being higher than the seed extract. The flavedo, from all the citrus fruits examined, contains more nitrogen than the albedo or carpellary membranes. The vesicles contain higher quantities of nitrogen than either the reamed juice or centrifuged juice. The navel of the green Navel oranges contains the greatest concentration of nitrogen in this fruit.

The aliquot required to give optimum results on the chromatogram was partially controlled by the presence of materials in the extracts such as acids and sugars. These constituents prevented the use of definite aliquots based on nitrogen content. The large aliquots required for the flavedo and albedo chromatograms indicate that the total free amino nitrogen, as compared to the total alcohol extractable nitrogen, is relatively low in these fractions. Approximately the same relation was found with the other citrus fruits tested.

In order to study whether concentration of the extracts at 35°C. under 28.5 inches of vacuum would significantly affect the observed differences in the free amino acid content of the extracts, representative extracts were concentrated at 0°C. under vacuum, the concentration requiring approximately 30 hours to complete. Chromatographic analyses of these extracts did not show any significant differences from the extracts concentrated at 35°C.

There is also the possibility of hydrolysis, ester formation, and alteration in amino acid structure occurring as the result of the acid pH in concentrating some extracts such as from lemon juice. Observation of the chromatograms of these extracts indicates that this is possible and that such solutions should be buffered at pH 7 before concentration.

The presence of a spot corresponding to α-amino-n-butyric acid in Navel tissues and not in other citrus tissues indicates that this amino acid may be a normal constituent in this fruit and not a breakdown product of an amino acid, such as methionine, as suggested by Dent *et al. (4)*. Underwood and Rockland *(23)* reported α-amino butyric acid to be present in California Valencia orange and γ-amino butyric acid in Valencia orange, Navel orange, Marsh grapefruit, and Eureka lemon juice as well as in other citrus juices.

Tyrosine was found to be associated with epidermal tissue. It was found in the Navel flavedo and albedo but not in other tissues of any of the citrus fruit examined, with the exception that it was present in the seeds of all the fruits examined. Its presence in these other tissues is probably of such low concentration that it could be detected using this technique of analysis.

It was found that in seedlings of certain higher plants the quantity of glutamine as well as of asparagine varied with the age and the environment of the plant. The more rapid hydrolysis of glutamine to glutamic acid as compared to that of asparagine to aspartic acid, may explain the consistent appearance of asparagine and the inconsistent appearance of glutamine, both qualitatively and quantitatively, Krebs *et al. (9)*.

Lysine and arginine, 2 basic amino acids, were found to vary both qualitatively and quantitatively. In a study of the basic substances in the alfalfa plant, Vickery *(24)* found that arginine was held partly in combination and that lysine was in total combination, that is, they did not occur free or as salts. Recently it was shown by Lea and Hannan *(10)* that lysine, arginine, histidine, methionine, and tyrosine could combine with reducing sugars and that under hydrolytic conditions only 70% of the lysine and none of the arginine or histidine could be liberated.

Free amino acids during browning. The nitrogenous constituents of citrus juices have long been suspected of being involved in the browning reaction, but Nelson *et al. (15)* found no nitrogenous constituent in aged pasteurized juice that was not present also in the fresh juice, and they reported that the amount of soluble nitrogen in aged juice was almost double that in fresh. Joslyn and Marsh *(7)* observed that the amino-nitrogen level remained practically constant during the course of browning of Valencia and Navel juices, and Loeffler *(12)* observed lower amino acid content in juices stored at 0°C. than those stored at higher temperature. Joslyn and Marsh *(7)* postulated the possible hydrolysis of polypeptides during storage to account for this. The isolation of an alkali soluble protein from the chromatophores of the orange by Smith *(19)* and later by Sinclair *et al. (18)* lent support to this hypothesis.

Reamed and centrifuged orange juice initially prepared and after 4 months of storage in half-filled cotton stoppered bottles was chromatographed, using the same amount of alcohol extract. The total alcohol soluble nitrogen content was 16.55 mg. per 100 ml. for the reamed and strained juice and 16.74 mg. per 100 ml. for the centrifuged juice. The initial ascorbic acid content was 48.4 mg. per 100 ml. for both samples. After storage for 2 weeks this decreased to 1.84 mg. in the reamed juice and to 11.9 mg. in the centrifuged juice, and after 4 months no reduced ascorbic acid remained in either. The extent of browning was followed by changes in optical density with 4 color screens in a Klett-Summerson photometer. In the reamed juice the photometer readings at 420, 540, 600, and 660 mμ, respectively, changed from 612, 294, 202, and 173, respectively, to 340, 61, 22, and 14. The corresponding readings for the centrifuged juice were initially 500, 253, 174, and 147 and after 4 months' storage 405, 78, 23, and 14. At this time considerable precipitate formed and settled out in both lots.

The extracts of the Valencia juice after 4 months of browning did not show any significant differences in the intensity of the number of spots. Further aging of the juice may bring out differences. This was observed in a one-year old Navel juice obtained from ripe market oranges. No noticeable change of free amino acid content was observed to occur during darkening. The confusion in the literature on changes in nitrogenous constituents during the browning of orange juice, Stadtman *(20)*, may be due in part to failure to include the reaction of reducing sugars with proteins such as that found by Lea and Hannan *(10)*.

As suggested by Joslyn and Marsh *(7)*, the hydrolysis of soluble polypeptides may be involved in the maintaining of constant amino nitrogen content during browning. This may be the explanation for the non-observance of quantitative changes of the amino acids in the paper partition chromatogram. Of notable importance is the observation that the centrifuged juice darkens over 3 times as fast as the reamed juice as measured at the 420 mμ absorption band.

Amino acids in orange proteins. The properties of the Valencia orange peel and chromatophore proteins are summarized in Table 4. The following

TABLE 4

Chemical tests on proteins

Test	Peel protein	Chromatophore	Constituent tested for
1. Color	Yellow	Cream white	
2. Total nitrogen (excluding NO_3)	4.35%	15.16%	
3. Xanthoproteic-confirmatory addition of alkali	Strong positive	Positive	Benzene nuclei, tyrosine, tryptophane, phenylalanine
4. Millons	Colored protein	Slight positive	Monohydroxy-benzene nucleus, tyrosine
5. Adamkiewiez	Negative	Positive	Tryptophane
6. Hopkins-Cole	Negative	Positive	Tryptophane
7. Molish	Strong positive	Very weak positive	Carbohydrate
8. Kovac's	Negative	Negative	Free indole nucleus
9. Cystine and cysteine sulfur	Negative	Negative	Cystine or cysteine
10. Sakaguchi H_2SO_4 hydrolysis	Positive	Positive	Free guanidine group
11. $Ba(OH)_2$ hydrolysis	Negative	Negative	Arginine

amino acids were detected in the barium hydroxide hydrolysate of the peel protein, in order of intensity of ninhydrin reaction: Lysine and possibly ornithine, unknown, aspartic acid, β-alanine, glycine, glutamic acid, alanine, proline, γ-amino butyric acid, tyrosine, valine, phenyl alanine, leucine, unknown. In the sulfuric acid hydrolysate the amino acids detected were: Lysine, unknown-arginine and ornithine, unknown, β-alanine, unknown, serine, glycine, aspartic acid, glutamic acid, threonine, alanine, proline, γ-amino butyric acid, tyrosine, valine, phenyl alanine, and leucine. The amino acids found in the barium hydroxide hydrolysate of chromatophore proteins were: Lysine, ornithine, unknown, aspartic acid, glycine, β-alanine, glutamic acid, alanine, proline, γ-amino butyric acid, tyrosine, valine,

phenyl alanine, leucine, and unknown. In the sulfuric acid hydrolysate of chromatophore proteins: Unknown, lysine, arginine and possibly ornithine, unknown, unknown, aspartic acid, serine and glycine, β-alanine, glutamic acid, threonine, alanine, proline, unknown, tyrosine, γ-amino butyric acid, valine, phenylalanine, and leucine.

Every amino acid spot found in the hydrolysate of the proteins isolated from the peel and chromatophores was present in the free amino acid extracts of orange juice, with the exception of glycine and possibly ornithine. Some amino acids, asparagine, glutamine and others, both known and unknown, were not found by either method of hydrolysis of the protein because of the destruction during hydrolysis. No significant differences were observed between the 2 proteins by chromatographic analysis.

The 2 proteins, whose isolation is described in this paper, were flocculated at pH 4.7, Smith *(19)*, the point of maximum insolubility of the chromatophore protein as determined by previous workers, Sinclair *et al. (18)*. It was found that precipitation actually occurred over a wide range of pH, the solution becoming cloudy at approximately pH 6.8 when adding acetic acid to the sodium hydroxide solution of the protein. The wide range on either side of the maximum insolubility of pH 4.7 suggests that a number of proteins may have been carried through the methods of purification.

The protein from the peel contains 4.35% nitrogen whereas the protein from the chromatophores has 15.16% nitrogen. In purification of the peel protein, the filtrate remaining after the protein was brought to the pH 4.7 and filtered out, was of bright yellow color. This color did not decrease or increase in intensity upon repeated precipitation and was not removable by washing the protein with water. On the other hand, the protein isolated from the chromatophores was almost white in color and the filtrate was colorless. The protein of the peel contained a substance which may or may not be in combination with the protein and which is fairly insoluble in the range of pH 4.7. The Molish reaction indicates this to be carbohydrate in nature. Sinclair *et al. (18)*, in their investigations of proteins of orange pulp, suggested that a similar carbohydrate material was pectin. The precipitation of pectin with the protein may be responsible for the occlusion of other impurities such as the ones responsible for the color and also for the fluorescence found in the barium hydroxide hydrolysis. The protein from the chromatophores is considered to be reasonably pure, if not altogether so. Its low Molish reaction may be due to chemically combined carbohydrate material. Separation of these proteins may be achieved by paper partition chromatography *(5, 13, 16)*, but this so far has not been applied. The use of the Waring blendor with its speed of approximately 10,000 r.p.m. caused some denaturation of proteins, principally enzymic protein, Stern and Bird *(21)*. Concentrated alcohol and ether also are known to denature proteins, but it is thought that their effect would have little influence on the amino acid constituents.

Glycine present in protein hydrolysates may be the result of hydrolytic decomposition. Serine was found to be stable in acid hydrolysis but destroyed in barium hydroxide hydrolysis, Lloyd and Shore *(11)*. We found the production of glycine in amounts over that present in the sul-

furic acid hydrolysis, indicating that glycine is a breakdown product of serine. If serine can decompose, it is reasonable to believe that threonine also is destroyed under the same conditions of hydrolysis. Arginine was found to be stable to sulfuric acid hydrolysis but was decomposed by barium hydroxide hydrolysis. This was confirmed by use of the Sakaguchi reaction. On the chromatogram the arginine spot disappeared and a new spot appeared when the proteins were hydrolyzed with barium hydroxide.

The concentrations of aspartic acid and glutamic acid were different by the 2 methods of hydrolysis, their concentration being low in the sulfuric acid hydrolysis and higher in the barium hydroxide hydrolysis. The decomposition of asparagine and glutamine will contribute to the concentration of aspartic and glutamic acids, respectively.

The basic amino acids were difficult to identify because of their changing mobility in the phenol solvent. Their R_f values are extremely sensitive to external conditions and to the constituents in the aliquot applied to the paper. Arginine was found to vary from a position coinciding with asparagine to that on the other side of glutamine, which is usually just above alanine. Fortunately, this varying R_f occurs only with the phenol solvent development and does not hold for the majority of the amino acids. Tyrosine was found to be present in both proteins as shown both by the chromatogram and by the Xanthoproteic and Millons reactions.

Although tryptophane could not be definitely located on the chromatograms, it was found by the chemical colorimetric tests to be present in the unhydrolyzed chromatophore protein, but not in the alkaline or acid hydrolysis of the protein. The addition of pure tryptophane to the protein hydrolysates did not give the location of this amino acid on the chromatogram. This would indicate that at the levels added or present normally in the protein hydrolysis, the tryptophane either was destroyed by the developing solvents or that it was over-riding another spot. The latter case seems unlikely because the use of a third solvent failed to show a tryptophane spot. Tryptophane could not be found in the proteins isolated from the peel, either chromatographically or by the Hopkins-Cole test.

The sulfur amino acids did not seem to be present in the proteins. They did not appear on the chromatogram, unless as methionine under valine, nor were cystine or cysteine indicated by their respective test. This does not eliminate their presence in low concentrations however and further investigations must be made in order to prove their absence positively.

Although histidine has been found in the chromatophore protein by previous workers, Sinclair *et al.* *(18)*, its presence in the unhydrolyzed proteins has yet to be determined.

SUMMARY

Paper partition chromatographic analysis of the alcoholic citrus extracts of late Valencia oranges, early green Washington Navel oranges, late grapefruit, and tree matured Eureka lemons showed the presence of the following free amino acids: asparagine, aspartic acid, glutamic acid, serine, threonine, alanine, proline, γ-amino butyric acid, valine, phenylalanine. The free amino acids which were found in some extracts and

not in others were lysine, arginine, glutamine, *a*-amino butyric acid, tyrosine, and one or more unknown members of the leucine group.

The proteins isolated from the peel and the chromatophores of the Valencia orange were believed to be identical with respect to the amino acids found.

No qualitative changes in the free amino acids occurred during the browning of the Valencia or Washington Navel orange juice.

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THE SUGAR AND ASCORBIC ACID CONTENT OF PAPAYAS IN RELATION TO FRUIT QUALITY[a]

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The quality of papayas on the retail market in Hawaii varies greatly. Successful commercial production of papayas in Hawaii requires the study of all factors which may affect the eating quality. This paper is confined to a preliminary report of the effect of season, and position on the tree upon the sugars and ascorbic acid in papayas and their relation to fruit quality. Organoleptic tests for color, texture, flavor, and sweetness of the fruit and assays for ascorbic acid content were made to determine if there might be any correlation with seasonal changes in the sugar content. This study should be of value in planning future work on papaya projects.

EXPERIMENTAL PROCEDURE

Description of fruit. The fruit were obtained from a papaya grove of Solo papaya,[c] variety Line No. 5, at the University Experiment Station farm in Manoa Valley containing 90 trees, of which 38 healthy, hermaphrodite trees were used for this project. The trees were planted in January, 1949, and the first fruit harvested in December, 1949. The data in this paper were obtained from 311 fruit harvested from February, 1950, through December, 1950, or a total of 11 months. In January, 1951, at termination of the project, sufficient fruit were not available for analysis because of poor fruit set six months previously. It is unfortunate that there was not a complete 12-month collection for the project.

Selection of fruit. Papayas were harvested twice weekly, Monday and Friday, when the apex of the individual fruit showed yellow. The directions of the fruit on the trees were noted with a compass and marked on each fruit as picked. Papayas picked when approximately one-sixth yellow and stored at room temperature 25°C.(77°F.) in semi-darkness, ripened to the optimum stage in 72 hours and were ready for analysis.

As a trial run, fruits from 25 trees, harvested from the same direction on the same day were desired. Because of irregular ripening it was necessary to select fruit from 2 directions on the same day, and to cover one direction of 25 samples over 2 analytical periods. Statistical analyses of the data for ascorbic acid and total sugars from 25 fruit in each of 4 directions, showed that a sample of 4 fruit from one direction at one harvesting was sufficient to collect data for final calculations. But a 7-fruit sample was used whenever possible to assure more than adequate sampling.

The plan was to test lots of 7 fruits for one of the 4 directions of north, south, east, and west each week and to cover a different direction in rotation. But due to the unpredictable physiological behavior of papayas, fewer samples were available some weeks when the fruit failed to set and mature. After 5 months, a second 25-fruit check-up in one direction was repeated and it was found that data collected from the regular, weekly 7 fruits constituted an adequate sample.

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[c] The Solo papaya is typified by the small, pyriform fruits borne on hermaphrodite trees. These fruits are approximately 6 inches long and 4 inches wide, and weigh about 1 pound. The Solo reaches maturity in 12 to 14 months, and first fruits are borne 4 to 5 feet from the ground. This fruit probably originated in British West Indies and was imported as seed into Hawaii. Because of its small size and excellent flavor, the variety quickly attracted attention as a fruit desirable both for home use and for the market *(6)*.

Each individual fruit was apportioned as follows for the organoleptic tests and chemical analyses: a one-third longitudinal section was reserved for tasting; a second longitudinal section was used for the ascorbic acid assay; and the remainder of each fruit was used for total sugar, moisture, and pH determinations. Only the edible portion of each fruit was used, and chemical determinations were made on samples blended in a Waring blendor.

Palatability scores. A panel of 4 to 5 experienced food judges scored the fresh papaya for color, texture, flavor, degree of sweetness, and general opinion. A possible high score of 5 and a low score of 1 covered the range for each factor.

The one-third longitudinal section of each fresh fruit reserved for tasting was cut into 5 approximately equal cross slices. The judges scored slices from the same position in each section of fruit throughout each test period. For example, judge number 5 always scored sample from position number 5 of each slice. All samples were tasted from the regular 7-fruit analysis periods, but only 10 samples chosen at random were scored by the panel on the check groups of 25 fruit.

Chemical methods. *Ascorbic acid:* Ascorbic acid was determined on a 20-gram edible portion cut from the section reserved for this analysis, using the Bessey and King method *(1)*, modified by Musulin and King *(4)*. Duplicate titrations were made on each sample. Results are reported as milligrams of ascorbic acid per 100 g. of fresh sample.

Total sugars: Total sugar determinations by the Munson-Walker method *(3)* were made on 50 g. of the sample blended for sugar, moisture, and pH analyses. Duplicate determinations were made on each sample. Results were calculated as percent total sugars on the fresh fruit.

Moisture: Duplicate determinations were made on two 5- to 10-g. portions of the blended sample which were dried in an air oven at 67°C. for 48 hours and finished in a vacuum desiccator for 8 hours. Results were calculated as percent moisture.

pH: The remainder of the blended sample left from the sugar and moisture determinations was used. Triplicate readings were made on each sample using a Beckman pH meter and glass electrode.

Statistical methods. Correlation coefficients and analysis of variance were calculated according to the methods recommended by Snedecor *(5)*.

RESULTS AND DISCUSSIONS

Color and palatability scores. In averaging individual scores of each taster for the section of the papaya reserved for the panel, very slight differences in scoring occurred from the stem to the blossom end. There were no noticeable progressive variations in scoring from the stem to the blossom end of the fruit slices, but it was noted that 2 members of the panel scored samples in all factors consistently lower than the other 3 panel members. Information from the present data does not justify stating that either the stem end or the blossom end of the papaya is preferable to the other.

Panel scores for color, texture, flavor, sweetness, and general opinion were analyzed to separate variations due to months, direction of fruit on the tree and to individuals. The analyses of variance showed significant differences due to months of the year for color, flavor, sweetness, and general opinion scores, but no significant differences due to direction of the fruit on the trees. Texture scores showed no significant differences for either months of the year or direction of the fruit on the trees. The factor for direction of the fruit on the tree was pooled with individuals in the final analyses below.

Panel scores	Source of variation	Degrees of freedom	Mean squares	"F" value
Color	Months of year	10	0.561	2.30[d]
Color	Directions within months and individuals	227	0.244	
Texture	Months of year	10	0.347	1.76
Texture	Directions within months and individuals	227	0.197	
Flavor	Months of year	10	0.970	2.02[d]
Flavor	Directions within months and individuals	227	0.480	
Sweetness	Months of year	10	0.536	1.93[d]
Sweetness	Directions within months and individuals	227	0.278	
General opinion	Months of year	10	1.249	2.32[d]
General opinion	Directions within months and individuals	227	0.538	

[d] Significant; p, less than 0.05.

The analyses of variance confirm the monthly trends in color, flavor, sweetness, and general opinion scores shown in Figure 1. It would appear that texture of the fruit is more uniform than the other factors studied and less subject to climatic influences.

The general opinion scores are separate scores just as color, flavor, texture, and sweetness scores. They are not average scores derived from the other 4, but rather an indication of overall rating of samples by each taster. All general opinion scores are noticeably lower in value than those for the other 4 factors studied, but consistently follow the trends of both flavor and sweetness scores (Figure 1).

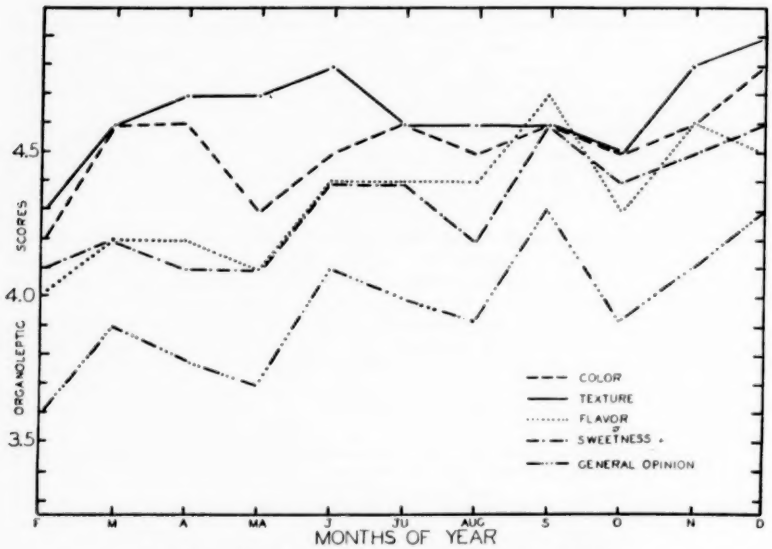

Figure 1. Mean organoleptic scores for fresh papayas.

When Line No. 5 Solo papaya was compared with 6 other varieties, the former scored considerably higher in the 3 factors of flavor, degree of sweetness, and general opinion, and slightly higher in color and texture of the fruit.

Taste scores for 21 samples of pistillate-type (female) papaya followed the same general pattern found in hermaphrodite fruit, but the scores for each taste factor were slightly lower in value. This type of fruit is not sold commercially because of its large seed cavity, small proportion of flesh, and rather firm texture when ripe.

Chemical changes. Mean monthly values for ascorbic acid, total sugars, moisture, and pH obtained over a period of 11 months from February through December, 1950, are presented in Figure 2. Each value was derived by averaging data collected in the 4 directions of north, south, east, and west, and covers from 14 to 50 samples analyzed per month, except in December when only 7 fruit were available.

The ascorbic acid values for 11 months present a range of variability from 87.3 to 105.4 mg. per 100 g. of fresh fruit. The highest values occurred in the months of March, July, September, and October; the lowest values during May and August. The abrupt drop in vitamin values for August warrants mention since it occurs between 2 months found to have high values (July and September). The direct causes for this sharp decline are not apparent from this study, but might be attributed to weather conditions when the fruit was setting on the tree, the amount of sunlight and rainfall during the ripening period, the temperature, and/or other factors.

Since the ascorbic acid of papayas is one of its important constituents, these results were analyzed to separate variations due to months, direction of fruit on the

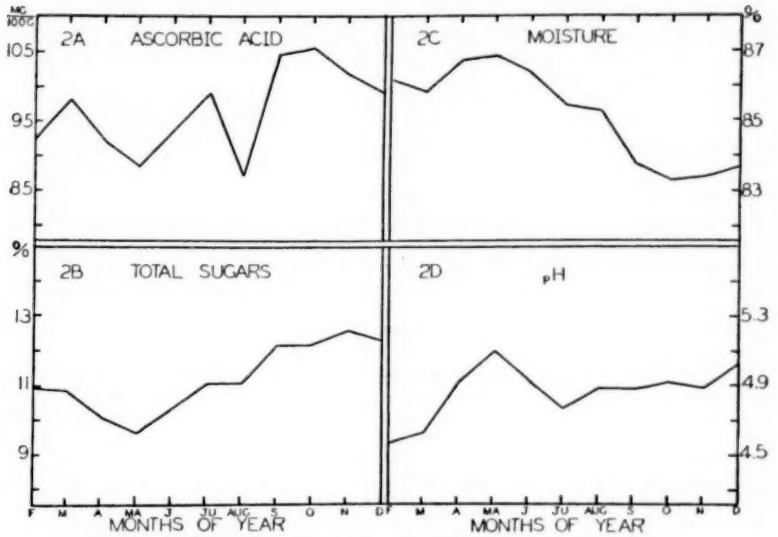

Figure 2. Mean values for chemical composition of fresh papayas.

2A—Ascorbic acid, mg. per 100 g.
2B—Percent total sugar
2C—Percent moisture
2D—pH

tree, and to individual fruits. A preliminary analysis of variance showed no significant variation due to direction of the fruit on the tree, so this factor was pooled with individual variations in the final tabulation. The complete analysis follows:

Source of variation	Degrees of freedom	Mean squares
Months of the year	10	775.48*
Directions within months and individuals	282	111.24

* Highly significant.

The differences in ascorbic acid due to months of the year were highly significant ($F = 6.97$; p, less than 0.01).

The highly significant coefficient of correlation (0.467) obtained between 234 paired samples of ascorbic acid and total sugar values appears to lend further support to the tenet that these 2 factors might be directly related as suggested by Miller *et al. (2)*.

From Figure 2c, the percent moisture within the fruit appears to vary inversely with the ascorbic acid values of the fresh fruit, and a highly significant correlation coefficient of minus 0.391 (234 paired samples) bears this out. This inverse ratio is most noticeable during the months of September and October when the percent moisture in the fruit declined.

In a previous study by Miller and co-workers *(2)*, ascorbic acid determinations on papayas from two University groves in widely separated locations showed high ascorbic acid values in April, May, June, and October. High values for this study occurred in May, July, September, and October.

Total sugars in Figure 2b, show a steady downward trend from February through May (11.0 to 9.7) and then a definite trend upward from June through November (10.4 to 12.3). Mean monthly values for total sugar and ascorbic acid generally

parallel each other with the exception of the month of August. A correlation coefficient of 0.808; p, less than 0.01 (234 paired samples) further substantiates this statement.

Analysis of variance of total sugar values as to months, directions, and individuals gave the following results:

Source of variation	Degrees of freedom	Mean squares
Months of the year	10	19.11[f]
Directions within months	24	0.98[f]
Individuals	235	0.53

[f] Highly significant.

As in the ascorbic acid analysis of variance, differences due to months of the year were highly significant ($F = 19.56$; p, less than 0.01). Principal factors contributing to these significant values are probably due to soil and genetic variations and to climatic conditions. These physiological factors would require specific equipment for direct measurement.

It is obvious from the table that variations by directions within months were such that they cannot be attributed entirely to chance ($F = 1.86$; p, less than 0.01). But the variation of directional mean values within months did not follow a uniform pattern, so there were probably interactions involved that might explain these differences. The physical collection of the data was not set up for interaction interpretation.

Correlation coefficients between total sugars and taste panel scores for flavor and degree of sweetness were found to be highly significant:

Correlation between	Number of paired samples	Coefficient of correlation, r
Total sugar vs. flavor	234	0.479[g]
Total sugar vs. sweetness	234	0.489[g]

[g] Highly significant.

Although these are "loose" correlations, they are highly significant (p, less than 0.01) and reflect the greater variability of the subjective scores over those of chemical analysis for total sugars. Individuals vary greatly in personal response and are also subject to error in judgment.

The pH values in Figure 2d show a slight tendency to vary inversely with the ascorbic acid values (r, minus 0.132; p, less than 0.05) and with the total sugar values (r, minus 0.250; p, less than 0.01). These correlation values are significant because of the large number of paired samples, but actually represent only very slight variation from their low "r" values. The highest mean value in May (5.11) corresponds to one of the lowest recorded mean values of the ascorbic acid (88.9), and the lowest mean value of total sugars (9.7). The pH values range from a low of 4.59 to a high of 5.11.

All the correlation coefficients calculated are relatively low, with the exception of the total sugar vs. ascorbic acid values, but because of the large number of paired samples, 234, they reflect certain trends over the 11 months covered by this experiment.

SUMMARY AND CONCLUSIONS

Over an 11-month period from February to December, 1950, 311 samples of Solo papaya, Line No. 5, were analyzed chemically for ascorbic acid, total sugars, moisture, and pH values. They were scored subjectively for color, texture, flavor, degree of sweetness, and general opinion by an experienced taste panel. The effects of season and position of fruit on the tree have been related to the results of both the objective and subjective factors in this study.

Ascorbic acid, total sugars, and the organoleptic scores of color, flavor, sweetness, and general opinion show significant monthly changes. Texture scores showed no significant differences by month. The best quality fruit

appear to be available from May through November when sugar and ascorbic acid content of papayas are highest. The problem merits further study, however.

Direction of the fruit on the trees within months was highly significant for total sugars, but not significant for ascorbic acid values or color, texture, flavor, sweetness, and general opinion scores. This presents another problem for future research.

Highly significant correlation coefficients were noted between total sugar and flavor scores, total sugar and sweetness scores, total sugar and ascorbic acid values, moisture and ascorbic acid values, and pH and total sugar values. Significant correlation coefficients were obtained between pH and ascorbic acid values.

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